The Generation and Analysis of Transgenic Mice Over-expressing the 5-Hydroxytryptamine (5-HT) Transporter.

Merewyn Katharine Loder

PhD

The University of Edinburgh

2003

Abstract

To explore the regulation and function of the serotonin transporter (5-HTT), transgenic (Tg) mice that over express the human 5-HTT were made. A 500Kb modified yeast artificial chromosome (YAC35D8) containing the human 5-HTT gene and its 5' regulatory sequence was injected into fertilized mouse oocytes, and Tg offspring were identified by PCR. Analysis by *in situ* hybridisation showed that h5-HTT mRNA is over-expressed in the cortex, hippocampus and mesencephalic raphe nuclei of these animals. RNase protection and RT-PCR showed that the human 5-HTT is expressed in these regions at levels comparable to, or greater than, that of the endogenous 5-HTT whilst the mouse 5-HTT RNA remained unaltered.

[^{3}H]-Citalopram, a high affinity 5-HTT ligand, was used to measure the protein expression. [^{3}H]-citalopram binding showed a higher K_D value ($P<0.02$) and a 2.3 - 3.5 fold increase in binding site density ($P<0.01$) in cortical membranes from Tg mice compared to controls. In addition, the Hill coefficient was less than 1 (consistent with the presence of two binding sites corresponding to the human and mouse 5-HTT).

Using HPLC, tissue extracts from the brainstem, hippocampus, midbrain, hypothalamus cerebellum and basal ganglia were analysed for 5-HT, DOPAC and 5-HIAA. The transgenic mice show a drop in 5-HT levels in all areas except the cerebellum (–17 to –40%) and a slight increase in 5-HIAA levels (6-17%); although this was only significant in the cortex (42% $p<0.05$). Thus the 5-HIAA to 5-HT ratio was increased by 27-76% in transgenic animals ($p<0.005$).

Irwin screening for behavioural abnormalities discovered no overt phenotype in transgenic mice. Mice were then treated with MDMA, a psychoactive chemical that acts at the 5-HTT, in a ramped dosing regime (1,3,10,30 mg/kg) one dose per day and horizontal, locomotor activity and body temperature recorded using telemetry equipment. Drug naïve transgenic mice were less active during the dark period of the diurnal cycle. There was a shift in the response curve for MDMA suggesting that transgenic mice have an increased sensitivity to MDMA. Transgenic mice showed an altered thermoregulatory response, with a pronounced hypothermia (reaching –8°C in one case) occurring within 7 hrs of administration of the 10 mg/kg dose of MDMA in contrast to a slight hyperthermia in wild-type animals.

In conclusion these mice show a moderate over-expression of the human 5-HTT, which results in significant alterations in the metabolism of 5-HT. This may provide a useful animal model of the neurochemical disturbances occurring in affective disorder in man.

I hereby confirm that the work in this thesis is all my own with the exception of the MDMA behavioural studies which were done in collaboration with Dr Hugh Marston, FINE, Edinburgh,.
None of this work has been submitted , in whole or in part, for any other degree program.

Acknowledgments

I would like to thank Professor Anthony Harmar for the honour of working on this project in his laboratory. Dr Harry Olverman and Paul Wren for all their help. To Dr Sanbing Shen for the lesson of independence and the new understanding of the role of tangential thought in science. To John Sheward for endless support and assistance above and beyond the call of friendship and Christine Morrison for a willing shoulder in times of stress.

Particular thanks go to Dr Trevor Sharp for allowing me to trade the bleak north for the rolling hills of the south, however briefly, and for reminding me of the importance of intellectual stimulation.

Finally, thanks to Diane Dinnis for eternal friendship and the strength to realise that things do get better.

Contents

ABSTRACT

ACKNOWLEDGMENTS

CONTENTS

CHAPTER 1. GENERAL INTRODUCTION **1**

1.1 THE 5-HT SYSTEM 2

1.1.1 5-HT synthesis *3*

1.1.2 5-HT metabolism *5*

1.2 THE 5-HYDROXYTRYPTAMINE TRANSPORTER (5-HTT) 6

1.2.1 The mechanism of 5-HT uptake *6*

1.2.2 Regulation of 5-HT transporter function *10*

1.3 5-HT NEUROTRANSMISSION 13

1.4 5-HT RECEPTORS 16

1.5 ANATOMY OF THE 5-HT SYSTEM 18

1.6 THE INDOLEAMINE/MONOAMINE HYPOTHESIS OF DEPRESSIVE DISORDERS 20

1.6.1 5-HTT expression in depression *22*

1.6.2 Effects of chronic SSRI treatments on 5-HTT and 5-HT *24*

1.7 TRANSGENIC AND KNOCKOUT MICE 25

1.7.1 Techniques for generating transgenic mice *26*

1.7.2 Use of transgenic mice as models *26*

1.7.3 5-HT and development *26*

1.7.4 Genetically modified mice and the 5-HT system *28*

1.7.5 5-HTT knockout (KO) mice *28*

1.7.6 The mouse model used in this research *30*

CHAPTER 2. THE MODIFICATION OF YEAST ARTIFICIAL CHROMOSOME 35D8 AND THE GENERATION OF 5-HTT TRANSGENIC MICE 32

2.1 INTRODUCTION 32

2.1.1 Modification of YAC 35D8 to include marker genes 32

2.2 METHODS 36

2.2.1 The Injection of YAC DNA into mouse oocytes and the generation of transgenic mice 36

2.2.2 Analysis of mice for presence of the transgene by PCR 37

2.2.3 Selection of Transgenic Mice and Colony Maintenance 38

2.2.4 Histochemical detection of β-galactosidase activity 40

2.2.5 Immunocytochemistry for HA epitope tag, β-galactosidase and 5-HT 41

2.3 RESULTS 43

2.3.1 PCR analysis, generation and selection of different lines 43

2.3.2 X-gal staining for Histochemical detection of β-galactosidase activity 44

2.3.3 Immunohistochemistry for β-galactosidase, 5-HT and Haemagglutin-tag in adult brains 49

2.4 DISCUSSION 49

CHAPTER 3 LOCALIZATION AND QUANTIFICATION OF RNA EXPRESSION IN THE BRAIN OF H5-HTT TRANSGENIC MICE 54

3.1 INTRODUCTION 54

3.1.1 5-HTT RNA expression in adult mice 54

3.1.2 RNA expression during development 54

3.1.3 RNA expression patterns and the study of gene expression 55

3.2 METHODS OF RNA ANALYSIS 55

3.2.1. Extraction of RNA from tissue samples 55

3.2.2 RT-PCR 56

3.2.3 RNase Protection assay 60

3.2.4 In situ *hybridisation analysis of RNA expression and localisation 62*

3.3 RESULTS 65

3.3.1. Tissue localisation of human and mouse RNA *65*

3.3.1.1 Tissue localisation in wild-type animals 65

3.3.1.2 Tissue expression in transgenics 67

3.3.1.3 Summary of RT-PCR results 70

3.3.1.4 Proportion of h5-HTT expression. 70

3.3.2 RNase protection assays *71*

3.3.3 Localisation and expression of mouse 5-HTT RNA in wild-type and transgenic mice *72*

3.3.3.1 Expression of Human 5-HTT RNA in transgenic mice 74

3.3.3.2 Expression of 5-HTT RNA outside the raphé 75

3.4 DISCUSSION 78

CHAPTER 4 PHARMACOLOGICAL CHARACTERISATION OF 5-HTT PROTEIN EXPRESSION 82

4.1 INTRODUCTION 82

4.2 METHODS 83

4.2.1 Membrane preparation from mouse and rat brain *83*

4.2.2 Preparation of rat platelet-rich plasma *84*

4.2.3 Preparation of membranes from rat and human PRP *84*

4.2.4 Protein assays *86*

4.2.5 [^{3}H]Citalopram binding assays *86*

4.2.6 Localisation of 5-HTT protein expression in the brain of transgenic mice by in vitro autoradiography with [^{3}H]citalopram *91*

4.2.7 In vitro autoradiographic [^{3}H]citalopram method and analysis *91*

4.3 RESULTS 92

4.3.1 Comparison of the pharmacological profiles of human, mouse, and rat 5-HTT. *92*

4.3.2 Affinity for citalopram and binding site density in the cortex and brainstem membranes of wild-type and transgenic mice. *97*

4.3.3 Pharmacology of antidepressant binding in the cortex and brainstem of wild-type and transgenic mice *101*

4.3.4 In vitro localisation of cortical [^{3}H]citalopram binding. *104*

4.4 DISCUSSION 106

4.4.1 Comparison of human platelet and mouse 5-HTT *106*

4.4.2 Comparison of transgenic and wild-type mice *107*

4.4.3 Summary *108*

4.4.4 Further work *109*

CHAPTER 5 CHARACTERISATION OF THE SEROTONERGIC NEUROCHEMISTRY OF h5-HTT TRANSGENIC MICE **110**

5.1 INTRODUCTION 110

5.1.1 5-HT and 5-HTT concentrations in knockout mice *112*

5.2 METHODS 114

5.2.1 Dissection of tissue samples *114*

5.2.2 HPLC detection of 5-HT, 5-HIAA and DOPAC *115*

5.2.3 HPLC detection of dopamine, DOPAC and 5-HIAA *116*

5.2.4 Data analysis *116*

5.3 RESULTS 117

5.3.1 5-HT and 5-HIAA concentrations in different brain regions of the mouse *117*

5.3.1.1 5-HT, 5-HIAA and 5-HIAA/5-HT concentrations 117

5.3.2 5-HT and 5-HIAA concentrations in h5-HTT transgenic mice *119*

5.3.3 5-HIAA/5-HT ratio and 5-HT turnover in h5-HTT over-expressing mice *119*

5.3.4 DOPAC and dopamine *119*

5.3.4 DOPAC and dopamine *124*

5.4 DISCUSSION 124

5.4.1 5-HT turnover and 5-HT concentrations *124*

5.4.2 5-HTT expression and depression *131*

CHAPTER 6 BEHAVIOURAL ANALYSIS OF THE RESPONSE OF TRANSGENIC MICE TO MDMA 133

6.1 INTRODUCTION 133

6.1.1 Behavioural studies in transgenic mice 133

6.1.2 5-HT and thermoregulation in mice 134

6.1.3 5-HT and dopamine interactions in thermoregulation in mice 136

6.1.4 Role of other transmitter systems and of corticosterone in thermoregulation 137

6.1.5 Responses of mice to MDMA exposure 137

6.2 METHODS 140

6.2.1 Irwin Screen 140

6.2.2 Telemetry apparatus 141

6.2.3 Dosing regime 142

6.3 RESULTS 143

6.3.1 Irwin screen 143

6.3.2 Baseline activity and temperature in transgenic and wild-type mice 145

6.3.3 Effect of MDMA on temperature in wild-type and transgenic mice 148

6.3.4 Effect of MDMA on locomotor activity in wild-type and transgenic mice 149

6.3.5 Other behavioural observations during the experiment 158

6.4 DISCUSSION 158

6.4.1 MDMA effects in wild-type mice 158

6.4.2 MDMA and locomotor activity in transgenic mice 160

6.4.3 MDMA and thermoregulation in transgenic mice 160

CHAPTER 7 SUMMARY AND CONCLUSIONS 165

7.1 5-HT KNOCKOUT MICE 165

7.2 THE 5-HTT OVER-EXPRESSING MICE 167

7.3 FUTURE WORK 170

7.4 USE OF TRANSGENIC MICE AS A TOOL FOR UNDERSTANDING THE COMPLEX INTERACTIONS OF THE 5-HT SYSTEM 171

7.5 CONCLUSION 172

REFERENCES 173

Chapter 1.

General Introduction

Genetic variability is an area of huge interest in the search for causes, treatments and prevention of disease, including disorders such as depression and anxiety. These disorders are believed to involve disturbances in the availability of neurotransmitters, particularly of 5-Hydroxytryptamine (5-HT or serotonin). An important aspect of the regulation of this transmitter is the specific 5-HT transporter protein (5-HTT), which removes the transmitter from the synapse. The expression of this protein can be influenced by individual genetic make-up or by experimental manipulation of the genome. Inserting or deleting specific sequences of DNA within the mouse genome creates an animal model for understanding gene function and the effects of enhanced or diminished expression of an individual gene. Such an animal model provides an excellent method to analyse the effect of changing one element in a complex system. Transgenic technology where protein expression can be moderately changed by inserting extra copies of a gene is a particularly useful and powerful tool for studying the ramifications of genetic variability on whole systems. In fact, one of the reasons that the 5-HT system is still poorly understood is because of its complexity. In addition to the transporter there are 14 known 5-HT receptor subtypes, which are expressed both pre- and post-synaptically.

Because the 5-HT transporter plays such an important role in regulating 5-HT, this study will examine the effects of increased 5-HTT expression in transgenic mice at two levels: locally on the 5-HT system and systemically on animal behaviour and physiology. A mouse model with extra copies of the gene for the 5-HT transporter may be particularly significant in view of recent evidence suggesting that the 5-HTT is increased in depressed patients (Dahlstrom et al., 2000).

The 5-HT system is well known, even to the general public, as the target of the antidepressant Prozac (fluoxetine) and because of its association with the effects, both positive and negative, of the popular recreational drug 'Ecstasy' (MDMA or

(±) 3,4-methylenedioxymethamphetamine). This study will therefore also examine the effects of increased 5-HTT expression on behavioural responses to MDMA.

The following pages of this introduction will present the areas of the 5-HT system most relevant to 5-HTT activity: 5-HT synthesis and regulation, the actions and regulation of the 5-HTT, 5-HT neuroanatomy and projections, the role of 5-HT and 5-HTT in relevant disease states, and the rationale for using a transgenic animal model.

1.1 The 5-HT system

In evolutionary terms 5-HT is one of the oldest transmitters in the human brain. The serotonergic system is involved in both neuronal development and in many functions of the central nervous system (CNS), including cognition, emotion, and possibly motor function. Serotonergic inputs from the raphé nuclei of the brainstem project throughout the forebrain and to the spinal cord.

The first identification of the substance now known as 5-HT (serotonin) was as a serum factor that caused vasoconstriction (Brodie, 1900). As a serum tonic factor, it was named 'serotonin' (Page, 1976). A substance found in the chromaffin cells of the enteric nervous system, which caused smooth muscle contraction (Vialli, 1933), was called enteramine. When isolated as the factor released from platelets during blood clotting, this substance was identified chemically as 5-Hydroxytryptamine (Page, 1976). The two substances serotonin and enteramine were found to be identical by Erspamer *et al* in the 1950's (Erspamer, 1963). Since the mid-twentieth century, the terms serotonin and 5-Hydroxytryptamine have been used interchangeably. The presence of 5-HT in the brain was first reported in 1953 by Twarog and Page (Twarog, 1988).

1.1.1 5-HT synthesis

The synthesis and metabolism of 5-HT is well documented and is tightly regulated (Figure 1.1.). 5-Hydroxytryptamine is synthesised from the essential amino acid tryptophan, obtained from the diet. The availability of tryptophan, which is transported across the blood-brain barrier by a neutral amine transporter (Blasberg and Lajtha, 1966), is a rate-limiting factor in the two-step process of 5-HT synthesis (Grahame-Smith, 1964; Hamon and Glowinski, 1974). First the 5' position of the indole ring of the tryptophan molecule is hydroxylated by tryptophan hydroxylase (TPH) in the cytoplasm of the cell, a process which requires NADPH, reduced pteridin, and oxygen as cofactors (Joh et al., 1975). The expression of this enzyme is specific to 5-HT neurons, and its activity is the rate-limiting step in the synthesis of 5-HT (Hamon et al., 1981). Second, the resulting product, 5-Hydroxytryptophan, is rapidly decarboxylated by the enzyme L-aromatic amino acid decarboxylase to form 5-Hydroxytryptamine (5-HT). This non-specific enzyme, which modifies most L-form amino acids, is found in high abundance in catecholamine neurons where it is also involved in the synthesis of noradrenaline (NA) and dopamine (DA).

Tryptophan hydroxylase activity is regulated at many levels (Joh, 1998). At the level of protein regulation, this enzyme's activity is regulated by phosphorylation of the non-active form of TPH to the active enzyme by both Ca2+/calmodulin-dependent (Hamon et al., 1981; Kuhn and Lovenberg, 1982) and cAMP-dependent (Foguet et al., 1993) protein kinases. The activity of cAMP protein kinases is dependent upon cAMP formation by adenylate cyclase, which is regulated by several 5-HT receptor subtypes, which in turn regulate 5-HT synthesis. 5-HT_{1A} receptors are negatively linked to both adenylate cyclase and to G_i-protein regulated K^+- or Ca^{2+} channels, and so may act through either protein kinase system (Starke et al., 1989; Williams et al., 1988). 5-HT_{1B} receptors are also linked to the adenylate cyclase second messenger system. The stimulation of 5-HT_{1A} receptors by agonists decreases 5-HT synthesis, but blockade of 5-HT_{1A} receptors only marginally increases 5-HT synthesis (Larsson et al., 1998; Stenfors et al., 2000). The terminal autoreceptor 5-HT_{1B} also inhibits 5-HT synthesis. Stimulation of the 5-HT_{1B}

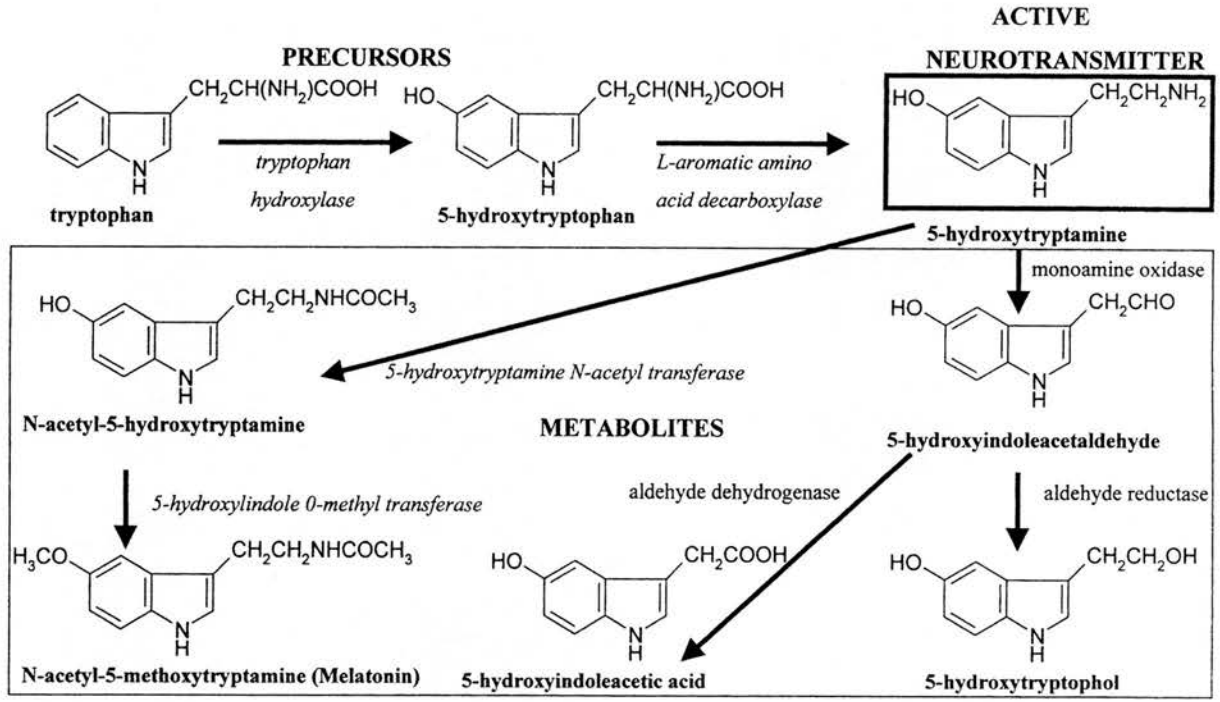

Figure 1.1 Pathways for Synthesis of 5-HT and its major Metabolites
(adapted from Wren 2000 from Joh (1998))

receptor markedly inhibits synthesis and the blockade of 5-HT_{1B} receptors increases synthesis, indicating that endogenous 5-HT does exert a tonic effect on synthesis via this receptor (Moret and Briley, 1997a; Moret and Briley, 1997b; Stenfors et al., 2000; Stenfors et al., 2001). Both 5-HT_{1A} and 5-HT_{1B} receptors can regulate synthesis independently under experimental conditions (Barton and Hutson, 1999), but they are probably not the only receptors involved physiologically. Selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants increase extracellular 5-HT and inhibit neuronal firing and 5-HT synthesis (Aghajanian, 1972; Carlsson and Lindqvist, 1978; Corrodi and Fuxe, 1969; Fuller et al., 1974; Gartside et al., 1995; Hjorth, 1993; Hjorth and Sharp, 1993; Hjorth et al., 1995; Invernizzi et al., 1992; Svensson, 1978). This acute effect is dependent on the presence of 5-HT and is not prevented by blocking 5-HT_{1A}, 5-HT_{1B} or a combination of both receptors (Moret and Briley, 1997a; Stenfors et al., 2001). This last observation suggests that another as yet undefined mechanism exists by which 5-HT release inhibits 5-HT synthesis.

1.1.2 5-H*T metabolism*

In addition to being regulated by changes in synthesis, 5-HT levels may be affected by changes in 5-HT metabolism. 5-HT is metabolised by the monoamine oxidase/aldehyde dehydrogenase pathway (Weissbach, 1961).

Of the two forms of MAO that are expressed differentially in the brain (Westlund et al., 1985), only the clorgyline-sensitive MAO-A is expressed in 5-HT neurons (Johnston, 1968). MAO-A, which is located on the outer mitochondrial membrane (Schnaitman and Pedersen, 1968), converts cytoplasmic 5-HT by oxidative deamination to 5-Hydroxyindole acetaldehyde. NAD^+-sensitive aldehyde dehydrogenase then converts the 5-Hydroxyindole acetaldehyde to 5-Hydroxyindole acetic acid (5-HIAA) the major metabolite of 5-HT (Duncan and Sourkes, 1974). A minor metabolite, 5-Hydroxytryptophanolcan, is produced from 5-Hydroxyindole acetaldehyde by metabolism by NADPH-sensitive aldehyde reductase (Cheifetz and Warsh, 1980). In the pineal gland and the retina 5-HT is also metabolised, via

N-acetyl serotonin, to produce melatonin, which is important in the co-ordination of circadian activity (Feldstein and Williamson, 1968; Klein et al., 1971). Regulation of 5-HT metabolism is thus largely dependent upon the activity of MAO-A, which is influenced by the availability of its substrate 5-HT, but enzyme activity may also be controlled by other feedback mechanisms. For instance, preliminary evidence suggests that MAO-A transcription and therefore 5-HT metabolism may be genotype specific. Human volunteers with a promoter polymorphism that increases MAO-A transcription have altered levels of 5-HT and dopamine metabolites in the CSF. This effect was genotype and gender specific with women showing increased monoamine metabolism whilst men had the opposite trend (Jonsson et al., 2000).

1.2 The 5-Hydroxytryptamine transporter (5-HTT)

The existence of the 5-HTT and other monoamine transporters has been known since the 1960's. These transporters are part of a Na^+/Cl^- dependent transporter family, usually referred to as the NET/GAT family after the first cloned proteins from this family (the norepinephrine transporter, NET, and the GABA transporter GAT) (Blakely et al., 1991). This family includes the proteins that transport the biogenic amines noradrenaline (NET), dopamine (the dopamine transporter (DAT), and 5-HT (the 5-HTT). These transporters, located on the plasma membrane, have a high sequence homology and a putative structure with 12 transmembrane domains (Figure 1.2).

1.2.1 The mechanism of 5-HT uptake

The recapture of released neurotransmitter via the plasma membrane is the first of two steps required for neurotransmitter recycling. Figure 1.3 outlines this uptake mechanism. The second step in the transport process sequesters cytoplasmic transmitters within vesicles via $VMAT_2$ in preparation for their release by exocytosis. A brief discussion of the biogenic amine transporters and the vesicular monoamine transporter (VMAT), which is involved in 5-HT transmission, is given below.

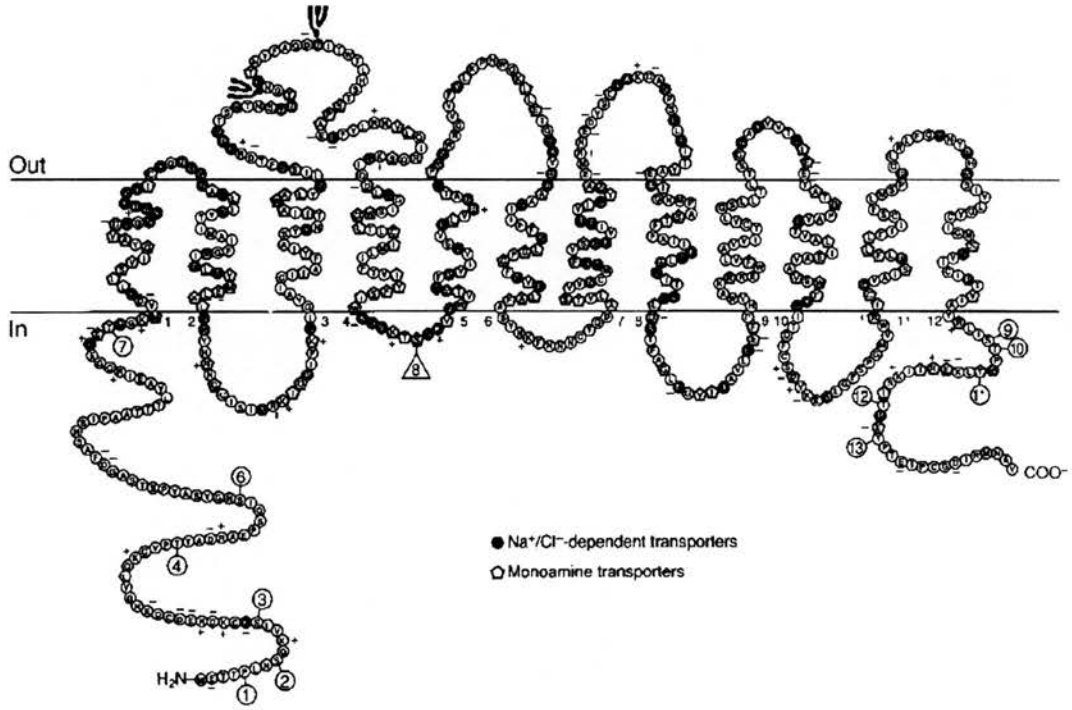

Figure 1.2 2-D representation of 5-HTT structure.

Potential Glycosylation sites are represented by "tree structures" on the 2nd extracellular loop. Numbers in circles represent potential phosphorylation sites. the number "8" in the stem and triangle structure is conserved among all monoamine plasma membrane transporters and reprents a protein Kinase C phosphorylation site. (Adapted from Benmansour et al 2002)

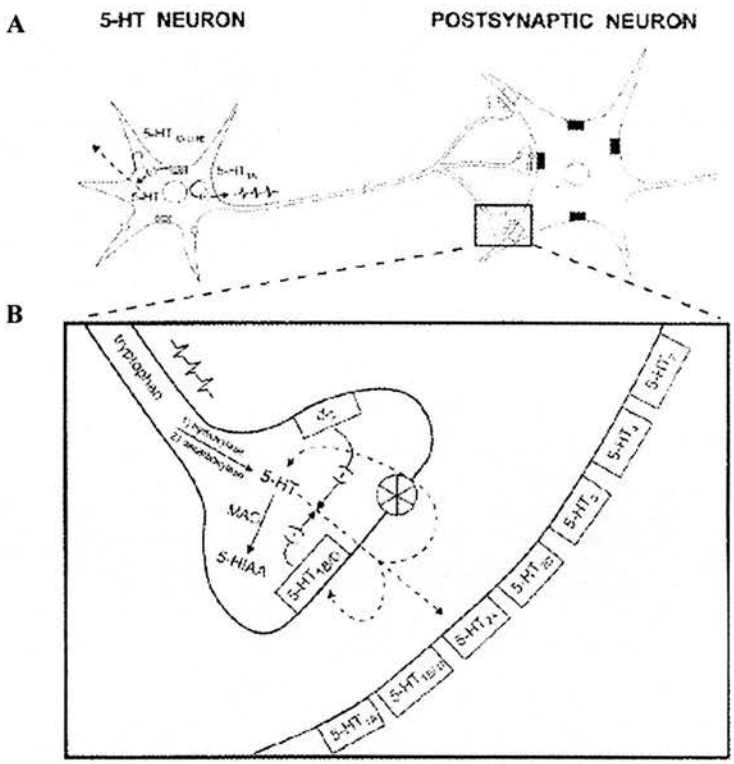

Figure 1.3 Diagram of a serotonergic neuron showing (A) factors involved in neuronal firing and somatic regulation and (B) synaptic transmission (adapted from Blier & De Montigny 1998)

As mentioned above, dopamine, noradrenaline and 5-HT are the main substrates for the DAT, NET and 5-HTT respectively, and these transporters are primarily restricted to their specific amine neuron type (Blakely et al., 1994; Lester et al., 1996; Lorang et al., 1994). All the monoamine transporters can transport other substrates in addition to their own .The DAT and NET transport their own substrates with an affinity of about 1μM though the NET can also transport DA with equal affinity. The 5-HTT has a slightly higher affinity for its substrate, in the range of about 300-500 nM. In addition to the endogenous substrates the monoamine transporters can transport neurotoxins such as amphetamine and MDMA. Various drugs such as cocaine and a wide range of specific inhibitors can also block transporters. Some inhibitors have a range of targets, for example Nomifensine has a high affinity for both DAT and NET. Desmethylimipramine (DMI) and nisoxetine however are selective for NET whilst Citalopram, clomipramine, paroxetine and fluoxetine are considered serotonin specific re-uptake inhibitors (SSRIs) due to their higher affinity for the 5-HTT. In addition there is some species variability in the affinity of inhibitors for the 5-HTT. These drugs will be used in this thesis research for pharmacological analysis of transgenic protein expression to confirm the expression of the human protein. This process is discussed in greater depth in Chapter 4.

Monoamine transport is Na^+ and Cl^- dependent and occurs by secondary active transport. The electrochemical gradient for this secondary active transport is generated by the plasma membrane Na^+/K^+ ATPase (Kanner and Schuldiner, 1987). The outwardly directed K^+ gradient coupled with the inwardly directed Na^+ and Cl^- gradients provides the driving force for 5-HT transport across the plasma membrane (Rudnick and Clark, 1993). The ionic dependence of monoamine transport appears to vary between transporters, at least in stably transfected cells (Blakely et al., 1994). Dopamine requires two Na^+ ions for co-transport, whilst noradrenaline and 5-HT use only one Na^+ ion. All three transporters also co-transport a single Cl^- ion (Blakely et al., 1994).

The most widely known theoretical model for transporter activity is that proposed by Rudnick and Clark (1993: Figure 1.4). These authors suggested that the 5-HTT forms two channel-like states, open and closed, which differ only in the accessibility of its central binding site. The model follows the gate-lumen-gate theory, behaving like a channel with a gate at each face, but only one gate can be open at any point in time. However, more recent evidence has suggested that there is more to the transport process than a simple carrier-mediated response. Electrophysiological studies using the dopamine transporter have uncovered a Na^+ current that the currents generated by co-transported ions could not explain.

Two different antidepressant-sensitive Na^+ fluxes were detected using both *Xenopus* oocytes (Lester et al., 1994; Lin et al., 1996; Mager et al., 1994; Zahniser et al., 1998) and mammalian cells (Galli et al., 1996; Galli et al., 1995; Galli et al., 1997). The one is associated with the transport cycle and the second with a leak current in the absence of substrate. Two reviews of these data (Lester et al., 1996; Sonders and Amara, 1996) suggest that transporters have a channel-like activity, possibly through the formation of a pore-like multimeric structure, whilst other evidence suggests that the transporter-associated leak current is due to both gates remaining open (Cao et al., 1998). The role of these currents in the transport process is still unclear and a fascinating area of investigation.

1.2.2 Regulation of 5-HT transporter function

The activity of the 5-HTT is regulated by extracellular factors such as amounts of 5-HT. 5-HTT levels are decreased (Benmansour et al., 1999) following prolonged treatment with SSRIs, possibly due to the effect of increased extracellular 5-HT, (Pineyro et al., 1994). Transporter activity can also be modulated by intracellular messenger systems, such as the protein kinase C pathway, which can be activated by neuronal receptors, and transcription factors such as retinoic acid receptors.

In human chorionic JAR cells, 5-HTT activity is modulated by chronic exposure to treatments that elevate cAMP, including cholera toxin (Cool et al., 1991). Inhibitors

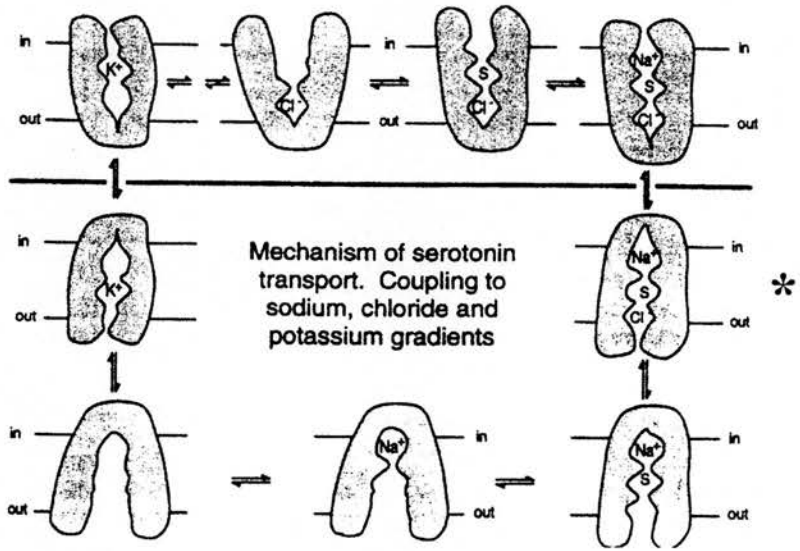

Figure 1.4 Standard Model for coupling of Na+,Cl-,and K+ ions to 5-HT transport

Starting at * 5-HTT binds extracellular 5-HT(s), Na+ and Cl- .In =cytoplasmic side, out =extracellular then converts to a form where the binding site for the ligand is accessible to the cytoplasm where 5-HT(s), Na+ and Cl- dissociate and K+ binds (figure adapted from Rudnick & Clark 1993)

of transcription and translation could prevent this effect, which had a delay of several hours. The human 5-HTT RNA increased in parallel with the 5-HTT protein, indicating a transcription-mediated increase (Ramamoorthy et al., 1993b). However, a non-cAMP-dependent pathway was also present (Lester et al., 1996; Ramamoorthy et al., 1995a; Ramamoorthy et al., 1995b; Sonders and Amara, 1996). In addition to regulation of transcription, 5-HTT function is regulated at the protein level. 5-HTT activity can be regulated by the activation of both protein kinase A and protein kinase C (PKC) pathways (Blakely et al.,1998; Qian et al.,1997). Platelet, endothelial and brain 5-HTT are down-regulated within minutes by PKC activation (Anderson and Horne, 1992; Myers et al., 1989). The down-regulation of 5-HT uptake in HEK293 cells by PKC occurs via a specific reduction in cell-surface protein (Qian et al., 1997). PKC induced down regulation could be due to phosphorylation of the transporter inducing protein translocation or to phosphorylation of other proteins causing alterations in intracellular trafficking. The 5-HTT has many putative phosphorylation sites on both the NH_2 and COOH termini and both termini can be phosphorylated by purified protein kinases suggesting a role for direct phosphorylation (Blakely et al., 1993; Blakely et al., 1998; Ramamoorthy et al., 1998). The rapid kinase-mediated regulation of 5-HT uptake activity, is concurrent with 5-HTT phosphorylation and is paralleled by reductions in 5-HTT surface abundance due to internalisation of the 5-HTT (Qian et al., 1997) suggesting a link between phosphorylation and internalisation. Recent evidence shows that 5-HT and other 5-HTT substrates inhibit PKC-induced phosphorylation of the 5-HTT and reduce loss of cell surface protein (Ramamoorthy and Blakely, 1999). This effect is abolished by the presence of antidepressants and may be a method to link 5-HTT expression to extracellular amine concentration. However recent studies on monoamine transporters also suggest that cell surface protein expression is more dynamic than previously thought and suggest the existence of additional, as yet undefined, regulatory mechanisms (Blakely et al., 1998).

5-HTT activity can also be regulated by rapid elevation/depletion of intracellular Ca^{2+} (Nishio et al., 1995), treatment with calmodulin inhibitors (Jayanthi et al., 1994), and by NOS/cGMP pathways (Launay et al., 1994; Miller and Hoffman,

1994). These alterations affect V_{max} of the 5-HTT activity, not its affinity for 5-HT, though parallel effects of modulators on vesicular amine pools may be occurring in these studies and obscuring measurements.

Transporter function is regulated in vivo via steroid hormones (McQueen et al., 1997) and pharmaceutically through the direct action of uptake inhibitors, which bind to a site on the 5-HTT that is closely related to the transmitter binding site, thus inhibiting transport (Graham et al., 1989). Chronic treatment with these agents probably causes a decrease in 5-HTT density and desensitises the transporter (Pineyro et al., 1994), as well as reducing 5-HTT mRNA levels (Lesch et al., 1993), suggesting action at the level of gene transcription. However, which intracellular mechanism meditates this action is unknown. Some evidence suggests that the 5-HT_{1B} receptor has a modulating effect on 5-HTT function via a direct G-protein mechanism (Daws et al., 2000). As with the regulatory effect of 5-HT, this modulation is important in the maintenance of 5-HT homeostasis (Daws et al., 2000).

1.3 5-HT neurotransmission

In a typical 5-HT synapse (shown in Figure 1.3) the 5-HT nerve terminal is found in close proximity to its postsynaptic target neuron. Upon firing 5-HT is released into the synaptic cleft from the presynaptic neuron, the signal is then terminated by transport of 5-HT back into the cell by the 5-HTT. Once returned to the cytoplasm the 5-HT is either degraded by the monoamine oxidase pathway or taken up into vesicles by the vesicular monoamine transporter (VMAT) where it is stored with the specific 5-HT binding protein (Levi and Raiteri, 1993; Rudnick and Wall, 1992; Tamir and Gershon, 1990). When the neuron is stimulated, the arrival of the action potential results in the depolarisation of the nerve terminal, which in turn causes the opening of voltage-sensitive Ca^{2+} channels and subsequent Ca^{2+} entry. The increase in intracellular Ca^{2+} induces exocytosis from secretory vesicles docked at the plasma membrane. However this exocytotic process is not the only mechanism for 5-HT release; another mechanism, particularly for release stimulated by drugs such as the amphetamines, is a carrier-mediated process. This process is Na^{+}- but not Ca^{2+}-

dependent and, unlike exocytosis, relies on a cytoplasmic rather than vesicular transmitter pool. Since this process is not modulated by terminal 5-HT receptors and is blocked by re-uptake inhibitors, it probably involves 5-HT transporter activity (Levi and Raiteri, 1993; Rudnick and Wall, 1992).

Vesicular monoamine transporters differ from the plasma membrane transporters in that they translocate transmitters from the cytoplasm into vesicles, against a concentration gradient, in preparation for regulated release by exocytosis. A single vesicular transport protein, the $VMAT_2$, is responsible for the re-uptake of all monoamine transmitters (DA, NA and 5-HT). The affinities of these transmitters for the $VMAT_2$ is similar, with 5-HT having a slightly higher affinity (0.2 μM; (Liu and Edwards, 1997). The $VMAT_2$ is also sensitive to reserpine and tetrabenzine (Liu and Edwards, 1997; Masson et al., 1999).

The VMAT couples efflux of two H^+ ions to the uptake of each molecule of 5-HT (Rudnick and Clark, 1993). The driving force for this vesicular transport is created by an ATP-driven H^+ pump, which acidifies the vesicle interior and creates a transmembrane electrical potential. The exchange of one amine substrate for the equivalent of two H^+ ions allows a 10-fold H^+ concentration gradient (one pH unit) to lead to a 100-fold gradient of neurotransmitter (Rudnick and Clark, 1993). This pH gradient is a stronger driving force than membrane potential, with only one charge crossing the membrane with each catalytic cycle (Rudnick and Clark, 1993).

As shown in Figure 1.3, 5-HT neurotransmission is a highly regulated process. The main autoregulatory effect of 5-HT on its own release is inhibitory. 5-HT is released from the terminal and the somatodendritic area of 5-HT neurons and has a profound effect on its own neurotransmission, via receptor-mediated regulation. 5-HT, released from the neuron or from axons of other neurons innervating the cell, activates receptors on the soma and on the synaptic terminal. These receptors induce a decrease in neuronal firing or somatodendritic release ($5\text{-}HT_{1A}$) or 5-HT release from nerve terminals ($5\text{-}HT_{1B/1D}$). The activation of $5\text{-}HT_{1A}$ receptors probably alters the propensity of a neuron to fire through altering the balance of currents

across the membrane. Indeed 5-HT_{1A} receptors have been shown to reduce a high-threshold Ca^{2+} current (Penington and Fox, 1994; Penington and Kelly, 1990; Penington et al., 1991) and to increase conductance to K^+ ions (Aghajanian and Lakoski, 1984; Penington et al., 1993). Stimulation of 5-HT_{1B} receptors, however, acts through second messenger systems to inhibit adenylate cyclase and consequently reduces cAMP thus inhibiting release. In addition to the autoreceptors, other receptors such as the alpha2-adrenergic receptors located on the 5-HT nerve terminals also inhibit 5-HT release (Araneda and Andrade, 1991; Maura et al., 1982). All of these factors contribute to the inhibition of 5-HT neurotransmission.

Once in the synaptic cleft, 5-HT interacts with a wide variety of postsynaptic receptors leading to a cascade of events involving second messenger coupling to intracellular mechanisms and resulting in either excitatory or inhibitory postsynaptic potentials. In some cell types more than one receptor subtype is present, sometimes with apparently opposite actions on neuronal activity. The paradoxical presence of two serotonin receptors mediating opposite effects on membrane excitability in the same cell provides a flexible mechanism by which serotonin might regulate neuronal activity. As an example, studies on cortical pyramidal cells exposed to extracellular 5-HT found that 5-HT simultaneously inhibited neurotransmission via 5-HT_{1A} receptors and stimulated activity *through* 5-HT_2 receptors resulting in an altered response to strong, but not weak excitatory stimuli (Araneda and Andrade, 1991). 5-HT neurotransmission is therefore influenced by many factors. Drugs can activate any of the 5-HT autoreceptors; drugs can block or alter 5-HT uptake (SSRIs) or vesicular transport (reserpine); monoamine oxidase can de-aminate 5-HT; and genetic factors can affect any of the variables involved. The 5-HT transporter is crucial as it is the only mechanism that terminates the 5-HT signal and recycles 5-HT; 5-HTT knockout reduces 5-HT concentrations by 65-90% of control (Bengel, 1998). The ramifications of over-expressing the 5-HTT on the 5-HT system are thus likely to be diverse.

1.4 5-HT receptors

A recently published article offers an excellent and comprehensive review of the 5-HT receptor field (Barnes and Sharp, 1999). The following is a brief summary of current knowledge with particular emphasis on the receptor types involved in regulating 5-HT neurons and which may be altered by overexpression of the 5-HTT.

All the known and well-defined 5-HT receptors, except for the 5-HT_3 receptor (Maricq et al., 1991), are from the G-protein coupled receptor family. The sequences of the many known members of this family vary significantly in their degree of homology with each other, but share a common feature: the presence of seven putative hydrophobic transmembrane domains. These domains have been implicated by site-directed mutagenesis experiments in specific agonist recognition (Chanda et al., 1993). The amino terminus is predicted to be external, whilst the carboxyl terminus is predicted to be intracellular. The carboxyl terminus, along with the predicted third intracellular loop, probably interacts with the G-protein and determines the recognition specificity. The binding of agonist to the receptor causes the heterotrimeric G-protein complex to dissociate into its subunits, the GTP-binding α-subunit and the $\beta\gamma$ subunit complex, both of which may interact with effector enzymes or ion channels within the cell to generate a response. Each receptor activates only certain types of G-proteins that will, in turn, influence only a subset of possible effector systems. The system activated by a particular receptor is therefore dependent on the G-protein and signal transduction mechanisms available in the cell type. This specificity is important as many of the signal transduction mechanisms postulated for 5-HT receptors are derived from experiments in heterologous expression systems and may not correspond to those seen *in vivo*. Table 1.1 summarises the second messenger systems of the 5-HT receptors (Albert et al., 1996).

Receptor	Second Messenger
5-HT_{2A}	Phospholipase C(+)
5-HT_{2C}	Phospholipase C(+)
5-HT_{2B}	Phospholipase C(+)
5-HT_{1A}	Adenylyl cyclase (-)
5-HT_{1B}	Adenylyl cyclase (-)
5-HT_{1D}	Adenylyl cyclase (-)
5-ht_{1E}	Adenylyl cyclase (-)
5-ht_{1F}	Adenylyl cyclase (-)
5-ht_{5A}	Adenylyl cyclase (-)
5-ht_{5B}	?
5-HT_{7}	Adenylyl cyclase (+)
5-HT_{4}	Adenylyl cyclase (+)
5-ht_{6}	Adenylyl cyclase (+)
5-HT_{3}	Ligand gated ion channel

Table 1.1 5-HT receptors and their second messenger systems

(+) represents stimulation (-) represents inhibition and ? represents unknown. Information taken from Barnes and Sharp (1999)

1.5 Anatomy of the 5-HT system

The neuroanatomy of the 5-HT system consists of two main pathways – the ascending and descending pathways, which originate in the raphé areas of the brainstem (Figure 1.5). Although 5-HT neurons represent a tiny proportion (<0.5%) of the total number of neurons in the brain, they project throughout the brain and as such are involved in many brain functions.

Originally the 5-HT containing cell bodies were classified into nine distinct cell groups (B1-B9) (Dahlstrom and Fuxe, 1964; Dahlstrom et al., 1965), which can be divided into two distinct divisions (Ungerstedt, 1971). B1-B3, the more caudal group, give rise to axons that descend to innervate the ventral and dorsal horns and the lateral column of the spinal cord. The rostral divisions in the raphé project to the forebrain and are most relevant to the neurochemistry analysed in this thesis. They consist of cell bodies in the caudal linear nucleus and the dorsal and median raphé nuclei (DRN and MRN, respectively), corresponding to B6-B9 (Tork, 1990). These nuclei have a wide pattern of innervation projecting to several different areas of the pre frontal and cerebral cortex as well as parts of the limbic system, basal ganglia and diencephalon (Steinbusch, 1981; Steinbusch et al., 1981).

Evidence from anterograde labelling and immunofluorescence studies indicates that in the rat, and almost certainly in the mouse and primate, the raphé nuclei have at least two populations of neurons (D and M), which differ in their areas of innervation, morphology and, nucleus of origin (Azmitia and Gannon, 1986; Molliver, 1987). The difference in morphology between projections from the two nuclei has been implicated in the apparent differences in neurotoxicity of chemical agents such as MDMA and p-chloroamphetamine (Gartside et al., 1997; Mamounas and Molliver, 1988; O'Hearn et al., 1988). Fibres that arise from the DRN are very fine, have frequently branching axons, and typically have small, pleomorphic varicosities that are granular or fusiform in shape (type D fibres). The D fibres project most heavily to frontal cortex and striatum, particularly to the associative and

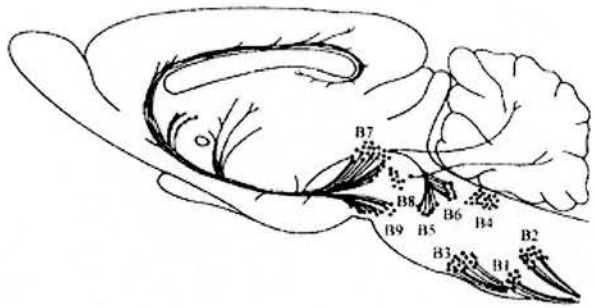

Figure 1.5 Schematic illustration of Major ascending and Descending 5-HT pathways in rodent brain

Cell groups B1-B9 represent the different raphe nuclei B3 project to the spinal cord . B5-B9 project to the forebrain. Most of the work in this thesis concentrates on the two nuclei which comprise the dorsal raphe (B6&B7)

B1=nucleus raphe pallidus; B2=nucleus raphe obscurus; B3=nucleus raphe magnus; B4=nucleus raphe obscurus; B5=median raphe nucleus; B6=dorsal raphe nucleus; B7=dorsal raphe nucleus (rostral); B8= caudal linear nucleus and nucleus raphe pontis; B9=nucleus raphe pontis and supralemniscal region (adapted from Wren 2000 from Cooper et al 1982)

limbic areas concentrated in layers III-IV (Azmitia et al., 1978; Jacobs and Azmitia, 1992). The fibres from the DRN are susceptible to both PCA and MDMA toxicity (Kosofsky and Molliver, 1987; Mager et al., 1994; Mamounas and Molliver, 1988; Molliver, 1987). In contrast axons arising from cells in the median raphé nucleus were found to be concentrated in particular areas of limbic cortex such as dentate gyrus, posterior cingulate, and entorhinal areas as well as in parietal cortex (Azmitia et al., 1978; Jacobs and Azmitia, 1992) and are characterized by large, spherical varicosities (type M axons) and by variations in axonal diameter. These coarser projections are more resistant to neurotoxins than the finer D fibres. As the mood-altering substances MDA, MDMA, and PCA act specifically upon 5-HT axon terminals from the dorsal raphé nucleus these neurons may be preferentially involved in the control of affective state (Kosofsky and Molliver, 1987; Mamounas and Molliver, 1988; Molliver, 1987).

5-HT projections have been reported from the B1-B3 regions of the rat and primate medulla via the ventromedial pathway to the ventral horn and the lateral pathway to the central grey area of spinal cord (Azmitia and Gannon, 1986; Dahlstrom and Fuxe, 1964). The 5-HT input to the spinal cord has been investigated using immunocytochemistry and the retrograde cell marker horseradish peroxidase (Bowker et al., 1982a; Bowker et al., 1982b) Whilst 5-HT immunoreactivity was found throughout the spinal grey area (most densely in the laminae I-IIa of the dorsal horn and laminae VIII and IX of the ventral horn), the major serotonergic input was from cell groups B1-B3, with B5, B7, and B9 having a smaller input to the cervical regions of the cord (Bowker et al., 1982a; Bowker et al., 1982b).

1.6 The indoleamine/monoamine hypothesis of depressive disorders

Recent technological advances in imaging techniques have made it easier to study the living human brain; however, they still have only limited efficacy and so most evidence for the role of 5-HT in depression is still circumstantial or based on model systems. The monoamine theory of depression, which was first suggested by Schildkraut in 1965, is based on the ability of reserpine to induce a depressive

syndrome and biochemical evidence such as changes in the levels of 5-HT and its metabolites in the cerebrospinal fluid (CSF) of depressed patients (Bryer et al., 1992; Martensson et al., 1989). This theory stated that depression was caused by a functional deficit of the monoamine systems and that mania resulted from an excess (Schildkraut, 1965). Several lines of evidence support this theory. Most drugs with antidepressant actions, including the earliest tricyclic antidepressants (TCAs), such as imipramine, block the reuptake of 5-HT in addition to their effects on other monoamines. It was then observed that the clinical efficacy of the TCAs was relative to their ability to inhibit the 5-HTT (DMI < imipramine < clomipramine) but not to the inhibition of other monoamine transporters suggesting that inhibition of 5-HT uptake might be responsible for the mood-altering effects of these drugs (Carlsson, 1969; Carlsson et al., 1969; Carlsson et al., 1968; Lapin and Oxenkrug, 1969). This observation led to the development of the indoleamine theory of depression, which hypothesized that 5-HT neurotransmission was decreased in depressed patients (Carlsson, 1969; Carlsson et al., 1969; Carlsson et al., 1968; Lapin and Oxenkrug, 1969) and led to the development of the most recent class of antidepressants, specific serotonin reuptake inhibitors such as fluoxetine. Further support for the indoleamine theory comes from studies of biochemical abnormalities in the CSF, blood or urine of depressed patients. Concentrations of 5-HT and its metabolite 5-HIAA have been found to be lower in the hindbrain of suicide victims suffering from depression, compared to sudden death victims (Lloyd et al., 1974; Shaw et al., 1967). Similarly, 5-HIAA concentrations were altered in the CSF of depressed patients (Dencker et al., 1966), particularly among those with a history of suicide attempts (Asberg et al., 1986; Coppen et al., 1967; Hertz and Sulman, 1968). Precursors of 5-HT, tryptophan and 5-Hydroxytryptophan (Coppen et al., 1967; Hertz and Sulman, 1968) have been shown to have antidepressant effects, suggesting that 5-HT availability is one important factor in depression. More recently studies on a subset of depressed patients in remission found that a low tryptophan diet caused immediate relapse into depression. This relapse was reversed by tryptophan supplementation (Delgado et al., 1990). Thus 5-HT availability an essential factor in at least some forms of depression. In depressed patients, neuroendocrine responses to L-tryptophan are altered (Price et al., 1991) and the hypothalamus-pituitary-adrenal axis (Calogero et

al., 1990; Lopez et al., 1997), which has been implicated in depression, is hyperactive. These effects are reversed by antidepressant treatment, but do not return to normal until clinical recovery is complete (Barden et al., 1995). 5-HT receptors, particularly those in platelets, have also been implicated in the pathophysiology of depression (Owens and Nemeroff, 1994). These 5-HT_2 receptors are increased in depressed patients whilst 5-HTT levels decrease. Both return to normal on clinical improvement (Owens and Nemeroff, 1994). Thus there is considerable evidence that alterations in the 5-HT system are symptomatic of depression and occur on a time course that does not preclude a causal nature.

1.6.1 5-HTT expression in depression

As antidepressant drugs act on the 5-HTT, considerable emphasis has been placed on the role of the 5-HTT in depression. 5-HTT levels in depressed patients have been measured by post-mortem autoradiography and by positron emission tomography (PET) studies in living patients. Both techniques have several problems. In post-mortem studies the tissue may be degraded, and the medical history and effects of drug treatments during life must be taken into consideration. An additional problem among various studies is the classification of depression, which varies from major depression to bi-polar disorder to suicide (Malison et al., 1998; Staley et al., 1998). It is therefore hardly surprising that the reported findings show little consensus. Several authors report reduced diffuse 5-HTT expression in the post-mortem forebrain tissue of depressed patients (see, for example, Mann et al., 2000), but other studies find no correlation between depression and 5-HTT levels in the DRN (see, for example, Bligh-Glover et al., 2000)

Recent innovations in PET and single photon emission tomography (SPET) scanning have allowed measurements of 5-HTT levels in living depressed patients (Malison et al., 1998; Staley et al., 1998). These authors found a reduction in the density of 5-HTT sites in the brainstem of unipolar depressed patients. Contrary to this study, (Dahlstrom et al., 2000) used SPET with ^{123}iodine-labelled 23-carbomethoxy-3P3(iodophenyl) tropane [^{123}I]beta-CIT as a tracer for monoamine transporters in

drug-naïve children and adolescents and found an increase in hippocampal/midbrain 5-HTT availability in depressed patients. The contrast between these studies can easily be explained by the possibility of prior drug treatment in the adult study and the different aetiologies for adolescent and adult unipolar depression. In addition, the use of radioactivity in the SPET studies restricted the available controls to other adolescents with psychiatric disease. Whilst this limitation was controlled for, it is always possible that a further factor such as dopaminergic disturbances complicated the result.

Two polymorphisms in the 5-HTT gene have been proposed to influence expression levels and to play a role in determining susceptibility to affective disorders. The first is a functional deletion/insertion in the promoter region (5-HTTLPR) with short and long variants (Heils et al., 1996), whose long form increases 5-HTT expression and whose short form reduces transcriptional efficiency (Lesch et al., 1996). Whether or not this polymorphism is significant is controversial; work from Lesch's group and another (Kunugi et al., 1997) have found a link between genetic status and affective disorder. The polymorphism has been linked in its short form with bipolar and unipolar disorders (Collier et al., 1996), bipolar only (Kunugi et al., 1997) and anxiety (Lesch et al., 1996). Post-mortem studies (Mann et al., 2000) have recently shown a link between major depression and the short allele. However, several studies have found no link between the short allele and affective disorder (Hoehe et al., 1998; Naylor et al., 1998) or 5-HTT levels in the human prefrontal cortex or hippocampus (Hoehe et al., 1998; Mann et al., 2000; Naylor et al., 1998). The contradictions among these studies may be explained by the small sample sizes used, which may have concealed significant findings. Evidence also suggests that the associations between 5-HTT expression and depression are race- (Kunugi et al., 1997) and gender-specific (Oruc et al., 1997), indicating the need for a large multi-ethnic analysis.

The second polymorphism in the 5-HTT gene is a variable number tandem repeat (VNTR) in the second intron, consisting of three alleles containing, respectively, 9 (STin2.9), 10 (STin2.10) and 12 (STin2.12) copies of a repetitive element. The short

9-copy allele has been linked to affective disorders, unipolar disorder more strongly than bi-polar (Battersby et al., 1996; Harmar et al., 1996; Ogilvie et al., 1996).. No evidence suggests that the VNTR would affect 5-HTT expression level (Battersby et al., 1996; Harmar et al., 1996; Ogilvie et al., 1996), though it may alter regulation.

1.6.2 Effects of chronic SSRI treatments on 5-HTT and 5-HT

The therapeutic effects of antidepressant treatments have a delay of weeks from the onset of treatment. It is therefore of interest to study the effect of long-term treatment with antidepressants on 5-HTT levels Although few studies have examined the effect of antidepressant treatments on 5-HTT function *in vivo*, considerable research has been done on the effect of chronic SSRI treatments on 5-HTT mRNA and ligand binding. The results of these studies are conflicting, with some studies finding increases, others no change, and some decreases (Benmansour et al., 1999). The reason for these apparent contradictions is probably the wide range of antidepressants and treatment regimes used in the early studies. Some drugs have a much shorter half-life than others, for instance citalopram has a half-life of 3 hrs. in the rat, whilst paroxetine has a half-life nearer one and a half hours. If the drug is applied by a single intraperitoneal injection every 12 or 24 hours, its serum levels may fall outside the therapeutic window.

Pineyro *et al* (1994) hypothesised that a continuous steady state exposure to a drug was required to alter 5-HTT expression level. More recent studies have taken this drug exposure into account. Even if a steady drug concentration is not required, it is still a better model for the situation in depressive patients where treatment regimes are designed to maintain a steady free serum concentration of drug within the therapeutic window. Benmansour (Benmansour et al., 1999) used an osmotic mini-pump to administer paroxetine, the most specific 5-HTT ligand available, sertraline, and DMI specific to the noradrenaline transporter, and measured the drug serum concentrations needed to maintain levels similar to those used therapeutically. Under these circumstances, SSRI treatment reduced 5-HTT ligand binding by 80-90% in the hippocampus, whilst 5-HTT binding increased by 50%. 5-HTT mRNA level was

altered only in the DRN, not the MRN. DMI or imipramine, both of which have a higher affinity for other transporters, reduced 5-HTT binding (Hebert et al., 2001), whilst fluoxetine reduced noradrenaline receptor and transporter expression. It would therefore appear that chronic lifetime treatment with 5-HTT inhibitors leads to down-regulation of transporter expression. This observation raises two interesting points. Does genetic knockout of the 5-HTT have similar effects on the rest of the 5-HT system as chronic SSRI treatment? Do genetic knockout studies thus offer a possibility for progress in depression research? As mentioned above, several studies suggest that polymorphisms that increase 5-HTT expression may be linked with depression. The possibility that 5-HTT overexpression causes the chemical imbalances of depression is therefore far from outrageous. Using 5-HTT knockout mice may be an effective tool for modelling the effects of chronic antidepressant treatments in humans.

1.7 Transgenic and knockout mice

The term transgenic is often used signify any manipulation of the genome. In this thesis, however, transgenic will be used only to define mice genetically modified to contain an additional DNA sequence, which is usually a gene from another species, most commonly human. These mice provide a way to study the effects of a single gene on development and behaviour in a whole system and so provide an excellent method for studying the expression and role of a gene throughout life. Since human disease may be caused by genetic factors that are present throughout life, transgenic mice provide a better model for studying genetic susceptibility to human disease when compared to pharmacological manipulations that are limited to a small window of time.

A knockout (KO) mouse is one that has been modified to remove a functional gene, either by mutation of the promoter sequence, which prevents gene transcription, or by removal of a coding exon to produce a non-functional transcript.

1.7.1 Techniques for generating transgenic mice

Extra DNA sequences can be inserted into the mouse genome through several mechanisms. The most common method is to inject a DNA construct containing the sequence of interest into the early-fertilized mouse embryo. A variety of DNA vectors may be used in such studies, but artificial chromosome vectors derived from yeast (YAC) or bacteria (PAC or BAC) have advantages since they permit very large DNA sequences, likely to include all of the regulatory DNA sequences of the gene of interest, to be expressed in transgenic mice.

1.7.2 Use of transgenic mice as models

Transgenic mice are extremely useful as models for gene function. They provide an *in vivo* system for studying changes in expression that occur throughout the life span and a method of analysing the function of single genes. However, there are several caveats to interpreting results from these studies. Firstly, there is always a possibility that inserting extra DNA will overtly alter other gene expression by accidentally knocking out another gene. Other more subtle effects on gene expression might include the inhibition of a promoter or regulatory transcription site or the disturbance of a regulatory system by altering the proximity of a modulatory site to its gene of action.

1.7.3 5-HT *and development*

The serotonin system is one of the earliest neurotransmitter systems expressed in mammalian development (embryonic day 12 in rats and days 11-12 in mice). The expression of this system at the same time as neuronal growth and differentiation has led to the suggestion that 5-HT may be involved as a signalling chemical for serotonergic development (Lauder, 1990). The projection of serotonergic neurons from the brainstem nuclei throughout the brain appears early in development. 5-HT has been shown to be mitogenic for both neuronal and other cell types and to control the growth and development of neuronal growth cones (Ivgy-May et al., 1994) in a highly controlled and protected manner. 5-HT has also been shown to induce

neurogenesis and neuronal differentiation (Lauder, 1993; Whitaker-Azmitia et al., 1996) affect cranial neural crest migration (Moiseiwitsch and Lauder, 1995), and induce synaptogenesis (Ivgy-May et al., 1994; Whitaker-Azmitia et al., 1996).

The serotonin transporter, 5-HTT, appears with a time course very similar to that of 5-HT (Zhou et al., 2000). The 5-HTT is expressed mainly in the raphé system, but also transiently in the cortex, striatum, substantia nigra and thalamus (Bruning and Liangos, 1997). Expression of the 5-HTT occurs before synapse formation, suggesting a role for neurotransmitter transport beyond signal regulation, which is similar to that seen in non-mammalian species (Lauder, 1993) where 5-HTT acts as a regulator of growth and motility. Moreover, it is likely that in brain areas where 5-HT is not produced, the transporter may be important for effects of 5-HT on cells there.

It is therefore likely that any factor which effects 5-HT uptake will have at least a subtle effect on the developing embryo. Given that SSRIs are known to cross the placental barrier and that many women take antidepressants during pregnancy, there is considerable concern as to their possible effects. It is also possible that a genetic predisposition to high or low levels of 5-HTT could alter neuronal differentiation, thus influencing the 5-HT or other transmitter system in later life and leading to a predisposition to depression and/or other diseases linked to 5-HT levels. In this case the presence of SSRIs during embryogenesis may have beneficial as well as negative effects.

Similar to 5-HTT knockout mice, the animals over-expressing 5-HTT produced for this research (see Chapter 2) do not show overt physical phenotypes, indicating that the increase in 5-HTT does not in itself control neuronal differentiation or cranial morphogenesis. Nevertheless, more subtle pharmacological and behavioural changes are seen in the mice that over-express the 5-HTT (see Chapters 3 – 6).

The increase in the 5-HTT, assuming it occurs throughout development as well as during adulthood, can be expected to increase 5-HT in areas of the nervous system where the transporter is the only means by which that area obtains 5-HT. The likelihood of a regulatory role for axonal and somatic 5-HTT makes its effect(s) on the 5-HT system more difficult to predict. Possibly the effect is counter-balanced by alterations in other parts of the system. As it is not yet known whether the 5-HTT has the same function in adults as in embryos, or even whether all components of the system such as vesicular storage are present, it is difficult to say whether an increase or decrease in 5-HT should be expected.

1.7.4 Genetically modified mice and the **5-HT** *system*

Many genes and proteins have been studied using transgenic and knockout mice. In the serotonergic system knockout mice have been created for the 5-HTT and for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2c} receptors. Mice have also been created to over-express the 5-HT_3 receptor, which is normally hard to detect in the CNS (Figure 1.6).

1.7.5 5-HTT knockout (KO) mice

There are now several strains of KO mice in which the 5-HTT is not expressed and a growing body of information about the effects of the gene knockout. Despite the importance of 5-HTT as a developmental signal, 5-HTT knockout mice survive with neither a physical phenotype nor anatomical changes compared to wild-type mice (Bengel, 1998). 5-HTT knockout mice have greatly reduced tissue 5-HT concentrations (60-80%) (Bengel, 1998). Removing the 5-HTT leads to several changes in 5-HT neuronal function. The 5-HT_{1A} autoreceptor, which controls neuronal firing, is desensitised (Gobbi et al., 2001) and reduced in expression in the dorsal raphé nucleus (Fabre et al., 2000; Li et al., 2000), whilst increased in the hippocampus and unchanged in the forebrain (Fabre et al., 2000). 5-HT_{1B} receptors are also site-specifically reduced in 5-HTT knockout mice, with reductions in the substantia nigra but not other brain regions tested (Fabre et al., 2000). The neuroendocrine and temperature responses mediated by 5-HT_{1A} receptors are also

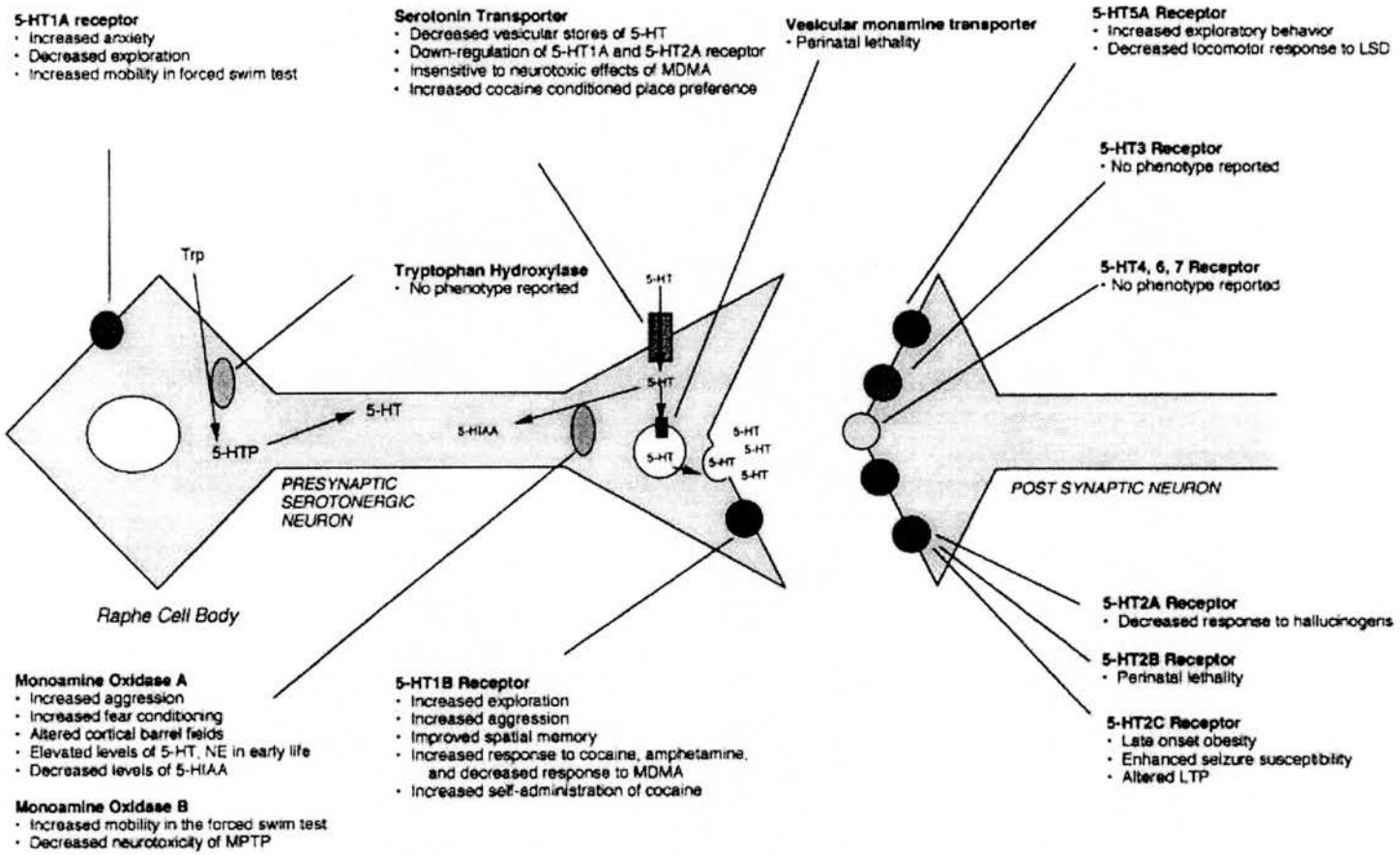

Figure 1.6 Summary of current knowledge of the effects of knockout of individual components of the 5-HT system on phenotype (adapted from Gingrich & Hen 2001)

diminished in knockout animals (Li et al., 1999b). In addition to changes in 5-HT_1 receptors, the knockout mice have altered 5-HT_2 and adenosine receptor expression. 5-HT_2 receptors are reduced in most areas, but with additional novel expression in the striatum (Rioux et al., 1999). Adenosine A1 receptors, which are involved in the regulation of 5-HT release, are altered in the DRN but not projection areas, whilst A2 receptors are altered in the basal ganglia (Mossner et al., 2000). 5-HTT knockout mice also show an altered response to the stimulatory effects of MDMA, but not amphetamines, with reduced locomotor stimulation in knockout mice. Paradoxically, increased activity was also seen in the heterozygote mice during the first 30 minutes after administration of MDMA. The 5-HTT knockout is of interest as a model for antidepressant treatments, where the 5-HTT is blocked. The therapeutic effects of antidepressants may be mediated by long-term changes in 5-HT levels induced by blockade of the 5-HTT, similar to those seen in the knockout mice. The changes that induce depression are not replicated in the KO mouse model. As explained in Section 1.6.1, evidence suggests that the 5-HTT is raised in some depressed patients (Dahlstrom et al., 2000). Mice that over-express the 5-HTT may provide a better model for the causes of the chemical changes inducing depression.

1.7.6 The mouse model used in this research

In the research described in this thesis, the transgenic technique is used to further study the role of the serotonin transporter by introducing the human 5-HT transporter gene into mice, so that they express greater than normal amounts of the 5-HT transporter protein. A yeast artificial chromosome (YAC) containing the human 5-HT transporter gene and regulatory regions was created (Chapter 2) and injected into mouse oocytes. The transgenic mice that developed from these oocytes provide a model for studying the influence of 5-HTT overexpression on the 5-HT system and behaviours associated with this overexpression, which has been suggested as a cause for depression in humans (Dahlstrom et al., 2000). The human transporter gene was used so that at a future date the transgenic mice could be crossed with knockout mice to produce animals with only human 5-HTT protein, thus providing a target for therapeutic drugs to be used in humans.

This chapter has provided an introduction to the 5-HT system, concentrating on the areas most relevant to the investigation, and to transgenic technology. Chapter 2 describes the production of the DNA insert and the generation of the mice. Chapters 3 and 4 describe the next stage, confirming that the inserted DNA leads to higher RNA and protein expression. Chapters 5 and 6 describe the functional consequences of 5-HTT overexpression on selected neurochemical and behavioural parameters of the 5-HT system.

Chapter 2.

The Modification of Yeast Artificial Chromosome 35D8 and the Generation of 5-HTT Transgenic Mice

2.1 Introduction

This chapter reports the modification of a yeast artificial chromosome (YAC) containing the human serotonin transporter (5-HTT) gene and the generation of transgenic mice containing this construct.

Yeast artificial chromosomes are a convenient way of inserting DNA into the genome of mice in a manner that is dependent on copy number but independent of position and with an expression pattern as similar as possible to the endogenous gene (Schedl et al., 1993b).

A YAC contains an insert of genomic DNA between short and long vector arms. The 500kb YAC 35D8 used here, identified by screening of the ICRF human YAC library (Shen et al., 2000a), contains the 40kb SLC6A4 gene which encodes the h5-HTT along with its upstream and downstream sequences. The upstream sequence contains the promoter region for the human gene which confers tissue specific expression, shown to be contained in 1.4kb of 5' flanking sequence (Heils et al., 1995) and so should confer a pattern of expression in transgenic animals that resembles that of the endogenous mouse 5-HTT. There are several polymorphisms found in the promoter region (see introduction). The YAC used contains the short form of the short/long promoter polymorphism and the 10 repeat allele of the intron2 9/10/12 polymorphism.

2.1.1 Modification of YAC 35D8 to include marker genes

Before injection into mouse oocytes the YAC 35D8 was modified. A short amino acid sequence (YPYDVPDYA) corresponding to the influenza virus haemagglutinin

(HA) epitope tag was inserted at the C-terminus of the open reading frame, together with the poly-adenylation sequences from SV40, the Yeast *ADE2* gene , and the *lacZ* reporter gene, which had been inserted downstream of a viral internal ribosomal entry site (IRES) (Mountford, 1994).

The addition of these sequences was done in an attempt to provide an easier method to analyse expression than the more traditional *in situ* hybridisation techniques (Vassaux and Huxley, 1997). The product of the *lacZ* reporter gene is the enzyme b-galactosidase, which can be detected with simple histochemical staining techniques. The HA epitope tag expressed at the C terminus of the 5-HTT protein can be detected with commercially available antibodies.

A two stage YAC manipulation system was used to generate transgenic mice by the method described in Shen *et al* (2000b). A brief description of the steps involved is given below. In the first stage, genomic sequences flanking the last exon of the 5-HTT were cloned around an HA-IRES-*lacZ-ADE2* cassette. After homologous recombination to incorporate the HA-IRES-*lacZ-ADE2* cassette into the YAC 35D8, in stage two an amplification vector pYAM4 was used to replace the long vector arm so that the YAC DNA could be amplified when grown in a medium containing galactose.

The two vectors used -YAC amplification vector pYAM4 and insertion vector pYIV2- are shown in Figure 2.1.2.1. pYAM4 is a derivative of pBluescript SK^- that contains a 572-bp SmaI-ClaI fragment linked to a conditional centromere (CEN4) that can be inactivated by induced transcription from an adjacent GAL1 promoter. pYAM4 also contains the yeast *LYS2* gene and the Escherichia coli hygromycin B resistance gene driven by the mouse *Pgk1* promoter. The amplification vector increases the copy number of inserted YAC per cell by inducing non–segregation of the YAC DNA when the GAL1 promoter is activated by the use of galactose rather than glucose in the growth medium.

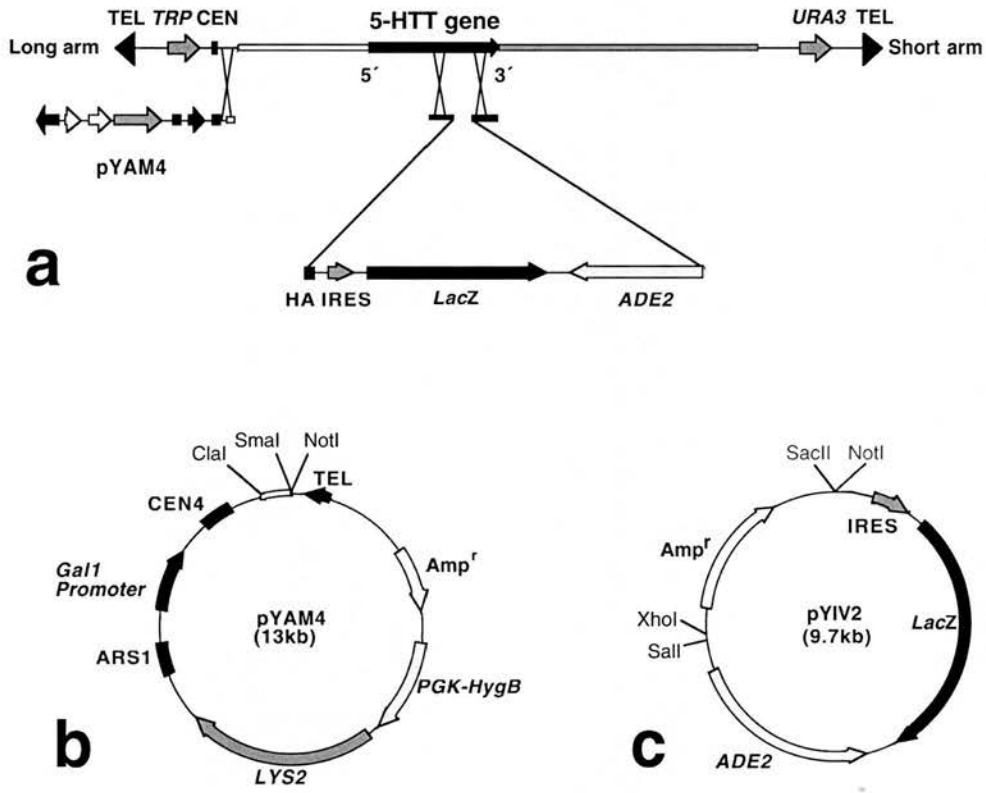

Figure 2.1.2.1 Two step genetic Manipulation of YAC DNA by homologous recombination (a) using YAC amplification vector pYAM4(b) and YAC insertion Vector pYIV3(c)

pYIV2 is a derivative of pGEM-11Zf that encodes the *lacZ* reporter gene. It is flanked by a viral IRES and poly-adenylation sequences from SV40 together with the Yeast ADE2 gene which in turn is flanked by unique restriction sequences to facilitate cloning. pYIV2 does not contain the amino acid sequence for the HA tag so this had to be inserted into the construct.

Stage 1 - The Insertion of the HA tag into the C terminus of the* 5-HTT *gene

The HA tag was introduced by inserting sequences from the 5-HTT gene into another construct which already contained the HA tag.

First a BamHI- XbaI fragment containing the $VPAC_2$ receptor with the HA epitope tag amino acid sequence (EYPYDVPDYASL) at the 3' end was blunt-ended and cloned into the EcoRV site of pBluekriptSK vector with a previously inactivated XhoI site to make plasmid **VIP2RHAskx**

Next, to subclone the 5-HTT sequences, a 5Kb human genomic DNA fragment containing intron 13 and exon 14 of the 5-HTT gene was cloned into the NotI-XhoI site of pBluescript (using PCR oligonucleotides ACT GCA TAG CGG CCG CAT CTT TCA TT GCA TCC CC and TGT GCT CGA GAG CAT TCA AGC GGA TGT) and then this was used to replace the NotI-XhoI $VPAC_2$ receptor fragment of VIP2RHAskx to make a vector containing only the 5-HTT intron13 exon14 and HA tag = **pIn13-HA.** The 5-HTT-HA sequence was isolated as a SacII-ClaI(blunt-ended) fragment and inserted into the NotI site of pYIV2 which contains the IRES-*lacZ-ADE2* sequence = **pIn13-HA-IZA**

In order to insert the sequence downstream of the stop codon, exon 14 of the 5-HTT was isolated by PCR (using PCR oligonucleotides CTC CTC GAG AGG AAA AAG GCT TCT and TAG GTA CC TGT TCT CTC CTA CGC AGT TT). It was then inserted into the XhoI-KpnI site of pBluescript to make **p3'**5-H**TT**.

Finally the intron 13 -HA-IRES-*lacZ*-ADE2 fragment was isolated by NotI and SalI digestion of **pIn13-HA-IZA** and inserted into the NotI and XhoI sites of **p3'5-HTT** to produce a construct containing the 5-HTT sequence from intron 13 with the HA-IRES-*lacZ*-ADE2 cassette inserted at the stop codon in exon 14 = **p*LacZ*5-HTT+** .

p*LacZ*5-HTT+ was linearised with NotI and transformed into yeast clone 35D8 by homologous recombination. The existence of the ADE2 sequence in the modified YAC 35D8/D6 was confirmed by Southern hybridisation with an ADE2 probe.

Stage 2 – The Replacement of the vector long arm with the amplification vector.
The modified YAC35D8/D6 was transformed with the NotI linearised YAM4 amplification vector. Ura+Ade+Lys+ recombinants were streaked on plates lacking tryptophan. Lys+Trp- clones were cultured in medium with 2% galactose. The YAC 35D8/D6 DNA was isolated and purified by pulsed field gel electrophoresis as described by Schedl *et al* (1993a).

In addition to this modified construct the unmodified YAC 35D8 containing the unaltered 5-HTT gene was used at a later date.

2.2 Methods

2.2.1 The Injection of YAC DNA into mouse oocytes and the generation of transgenic mice

Mice were generated by a variation of the method of Schedl *et al* (1993a). This method involves the direct injection of purified, modified YAC DNA into the male pronucleus of fertilized oocytes. The DNA is incorporated into the genome when the male and female pro-nuclei fuse. This method has the advantage over the use of the unpurified yeast genome of easy incorporation of only the target DNA rather than the entire yeast genome and, unlike methods where DNA is inserted into embryonic stem cells, generates non-chimeric animals. The large size and low concentration of YAC in the injected solution usually limits the number of copies of the YAC that are

incorporated, and so the transgenic animals generated usually contain only one or two copies of the gene of interest.

CBAxC57BL6 F1 female mice (CharlesRiver, UK, Margate, Kent) were superovulated and mated 24 hours later. Those which plugged successfully were killed by cervical dislocation and the fallopian tube and ovary were carefully dissected and cleaned and placed in M2 medium (Sigma). At this stage of pregnancy the fertilized oocytes are still in the upper part of the fallopian tube and can be removed by gently tearing the tissue wall where the tube is swollen or gently expelled by inserting a mouth pipette into the end of the tube and blowing through.

Fertilized oocytes were cultured for a few hours in M2 medium (Sigma) at 37 °C before injection. The solution containing the YAC was then micro injected into the male pronucleus using an Axiovert 100 microscope (Carl Zeiss) and automatic injector and manipulators (Narashige) with instrument holders from Narashige and Eppendorf.

The injected embryos were cultured overnight or for a few hours in medium M16 (Sigma) and living embryos (those which had completed cell division) gently inserted into the fallopian tube of pseudopregnant CD1 female mice under general anaesthetic. About 30 embryos were inserted per mouse using a mouth pipette and a dissecting microscope attached to a video camera and monitor. Mice were allowed to recover from the anaesthetic and pregnancy continued. Birth occurred naturally. Offspring were allowed to reach weaning age when a sample of tail tissue was taken and transgenic animals identified by PCR.

2.2.2 Analysis of mice for presence of the transgene by PCR

Genomic DNA was extracted from samples of tail tissue by digestion with proteinase K (6mg/10ml) (Boerhinger Mannheim) in a lysis buffer (600µl per sample of 50mM Tris-HCl pH 8, 100mM EDTA, 100mM NaCl, 1% SDS) overnight at 60°C. The digested sample was spun to remove remaining debris such as hairs and the

supernatant containing the DNA precipitated in a fresh tube with 0.8 volumes of isopropanol. Samples were mixed and DNA was precipitated for 20 minutes at room temperature followed by centrifugation at room temperature for 5 minutes. The resulting DNA pellet was washed with 70% ethanol and the DNA was re-suspended in 100 or 200 µl of PCR grade water by incubation at 60°C for 2hrs. or overnight at 4°C and mixed before being used in PCR reactions.

To identify transgenic mice, DNA samples were tested by PCR analysis with several primer pairs. Primer sequences and conditions are listed in Table 2.2.2.1.

Each mouse was first analysed with primers specific to the 5-HTT gene (exon 1A, exon 1B and 3'UTR). To check how much of the construct was incorporated, the mice positive for the 5-HTT gene were then tested with primers for the STS markers near the gene; D1S1294, WI1570, (Shen et al., 2000a) and the long and short arm of the YAC. All primers were obtained from MWG Biotech AG.

The same primers were used to identify transgenic animals throughout this work and to genotype the embryos used in the histochemical X-gal staining.

2.2.3 Selection of Transgenic Mice and Colony Maintenance

Those animals positive for YAC DNA were given specific 4 digit numbers in the form A10x.x. Each animal was bred with a CBA/C57BL6 wild-type animal and all offspring of this animal (referred to as a transgenic line) was given the founder's "A number" in addition to their own identifying number. The offspring were tested as above. All the lines were found to breed and produce transgenic offspring in the expected proportion of about 50% of the offspring. The lines which did not contain the entire 5-HTT gene were discarded.

Breeding was continued to provide sufficient animals for experiments and to establish a colony. Wherever possible breeding pairs were 3-6 months in age. All experiments were conducted on heterozygote animals to avoid the possibility of

	Code	Forward primer	Reverse primer	Melting temperature (°C)	Extension time	No. of cycles
HYGROMYCIN	A	TCT ACA CAG CCA TCG GTC CAG ACG	TTG ACT GGA GCG AGG CGA TGT TCG	66	30secs	35
5-HTT $_{1A}$ promoter	B	GCG TCT CGG TGG CAC CAG AAT C	TCG CGC TTC TGT TCC CAG CTA C	60 *	1min	30
5-HTT Exon 1B	C	CTA GTC ACT GAC ATT GCC TGG	TGT CCA GTC TAT CTG CAC ATG	56 *	30secs	35
5-HTT 3' untranslated	D	AGT TCT GAT GAG GCA GCG C	TTCATCACC TCC ATC CAC ATCC	55	1min	30
D17S1294	E	TGGCATGCAATTGTAGT CTC	TTCTTTCCTTACTAAGT TGAGAACG	56	1min	35
D1S1594	F	GAA TAC CAG GTC ACC ACA TGG	AAA CT TG ACA GGG TCT TG	55	1min	30
D1S2009	G	TGGCTTTAGTATTTTT CTTTGTTTT	GAAAAGTACTCCTCA GTAGGTTGAA	52	30secs	30
YAC short arm	H	TGT TAC TTC TTC TGC CGC CTG C	TCT CCG AAC AGA AGG AG AAC G	60	1min	35

Table 2.2.2.1 Oligonucleotide sequences and conditions for PCR analysis of 5-HTT transgenic mice.
Conditions listed above are for the main reaction. All reactions had a preliminary 5 cycles at 2°C higher than the melting temperature above to minimise false positives. *= hot start PCR. Temperatures were calculated according to the formula:
T_m=69.3 + (41x (No. of GC/total no. of bases)) – (650 / Total no. of bases) and conditions adjusted as necessary.

accidental knockout. Accidental knockout occurs when the injected DNA incorporates into the middle of another gene and prevents its expression. Homozygote animals will contain no functional copies of this endogenous gene and the gene is therefore "knocked out". Any phenotypical effects may be due to this lack rather than the over expression of the injected DNA. This occurred in the generation of the MAOA knockout mice which were produced when an injected interferon gene was incorporated into the MAOA locus (Cases et al., 1995). However, breeding for h5HTT homozygotes was carried out and the offspring were crossed with a wildtype animal to test for homozygosity. Homozygotes were produced in the ratio expected and bred well. Therefore this is not a life threatening modification.

2.2.4 Histochemical detection of β-galactosidase activity

The *lacZ* gene incorporated into the construct should be expressed in the same areas as the 5-HTT (Vassaux and Huxley, 1997). Its product β- galactosidase can be detected by its activity in cleaving X-gal (5-bromo-4-chloro-3indoyl-B-D-galactside) to produce a precipitable blue-green product.

CD1 female mice were mated with heterozygote male transgenic mice as CD1 animals produce larger litters than F1 animals. The mice were then culled at 12.5, 13.5, 14.5, 18.5 days post fertilisation (E0.5 = date of plug) and the uterus removed and placed in ice-cold Phosphate Buffered Saline (PBS). Embryos were carefully removed from the uterus and washed in PBS in a 24 well dish to remove contaminating fluids. Embryos were then fixed in 4% Paraformaldehyde E12-13 for 3hrs., E14-17 for 4hrs., larger embryos and newborns overnight. A sagittal cut was made down the midline of large embryos for ease of access to the staining media. After washing for three periods of 20 minutes in PBS containing 0.01% sodium deoxycholate (pH adjusted to 8.4 to minimise background staining)) each time, embryos were transferred to filtered Xgal (5-bromo-4-chloro-3indoyl-β-D-galactside) staining solution (1mg/ml X-gal, 5mM $K_4Fe(CN)_6$, 5mM $K_3Fe(CN)_6$, 2mM $MgCl_2$, 0.01% sodium deoxycholate 0.02% NonidetP-40 in PBS) filtered

through a 0.2 Å filter. X-gal was dissolved in DMSO at 40mg/ml. The final concentration of DMSO in the final solution was less than 1%.

Tissues were incubated at 30°C overnight with gentle shaking for the larger embryos. The staining solution was then removed and the embryos washed in PBS for a minimum of 2 hours. After staining the embryos were transferred to increasing concentrations of glycerol: 40% 10 minutes, 80% 10minutes, 80% overnight to clear then examined and photographed using a Contax 167 MT camera and Kodak colour film.

New-born pups were culled by schedule one method – Pups were injected with an overdose of sodium pentobarbitone followed by insertion into ice-cold PBS to preserve the tissue - then fixed and stained as for 18.5 day embryos.

The pictures used in this chapter are reproductions of the slide obtained by scanning in combination with Adobe PhotoShop ® software (Adobe systems Inc)

2.2.5 Immunocytochemistry for HA epitope tag, β-galactosidase and 5-HT

Mice were killed by injection of a lethal dose of sodium pentobarbitone with slow perfusion of about 50 ml of ice-cold 4% paraformaldahyde in PBS to fix the tissue. The brains were then dissected rapidly and postfixed for 2 hours at 4°C in paraformaldehyde (4%) / glutaraldehyde (0.05%), washed briefly in PBS and incubated overnight in 30% sucrose before sectioning. 30 micrometer sections were cut on a freezing microtome and thaw mounted on slides. The antibodies and conditions used for labelling are listed in Table 2.2.4.1

In general sections were allowed to dry for about 30mins then blocked for 30 minutes at room temperature in goat serum diluted in PBS with 0.1% Triton X-100. After blocking the solution was removed and the slides blotted before the primary (1°) antibodies were added. The 1° antibodies were diluted in fresh blocking

1° antibody	Source	1° antibody concentration	Blocking solution	2° antibody concentration
β-Galactosidase	Rabbit (5 Prime-3Prime, Inc. USA)	1/100 1/200	10% goat serum + 0.1%triton X in PBS	1/200
Hemagglutinin tag	Rabbit	1/200 1/500	1% goat serum + 0.1%triton X in PBS	1/200
5-HT	Rat IgG (Chemicon)	1/1000 1/2000	1% goat serum + 0.1%triton X in PBS	1/200

Table 2.2.4.1 Conditions and antibodies used for immunocytochemical analysis of 5-HT, haemagglutin tag and β-galactosidase activity in wildtype and transgenic mice. 1° = primary; 2°= secondary

solution and incubated overnight at 4°C. Incubation was in sealed boxes containing paper saturated in PBS to prevent the slides drying out

Slides were then washed in PBS for 3 times 30 minutes at room temperature, then incubated in the secondary antibody for 1 hour at room temperature, then washed 3x 30 minutes in fresh PBS before mounting in xylene.

Fluorescence was viewed using an Axiovert 135M inverted microscope (Carl Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK) with x40 objective. Immunofluorescence was observed using an appropriate filter under UV light (395nm). Antibodies tagged with FITC emitted at a wavelength of 509nm Fluorescence was photographed with a Contax 167MT camera attached to the microscope, using the appropriate exposure time and Ektachrome 160T (Kodak, UK) colour slide film.

2.3 Results

2.3.1 PCR analysis, generation and selection of different lines

298 oocytes were injected with YAC 35D6 and inserted into CD1 mice. Of these 97 (32%) were born, of which 6 died, and the rest were tested for the presence of the YAC transgene.

18 of the 97 offspring produced were positive for some part of the YAC (18.6%). Three founder mice were found to contain the complete YAC (A102.3, A102.5 and A105). Although this does not rule out splitting of the chromosome during injection, it makes it unlikely. Shearing forces, which occur during injection, can cause the large YAC to split into several sections that incorporate into the genome in different places. In this case the majority of the DNA will be present but non functional, it also leads to the loss or break of the sequence. If a DNA marker is not detectable it is probably due to this splitting and those animals will not express the human protein. Therefore only animals which contain all the 5-HTT gene markers are likely to

express the protein. Table 2.3.1.1 shows the PCR results. One of the animals had only one area of the 5-HTT gene and so was culled. Those without 5-HTT gene incorporation were discarded and work concentrated on those lines with the greatest incorporation (meaning most complete YAC-in which all the primer sequences were detected) (Figure 2.3.1.1).

Transmission to the next generation (germline transmission) was obtained from all the lines maintained. However it became impossible to breed all the remaining lines due to constraints in the breeding facility so lines that did not contain the complete gene were also discarded.

Lines A102.3 A102.2 A102.5 A101.2 A104 were maintained in the breeding programme whilst others were frozen as sperm for use in future studies.

As described below *lacZ* was not detected in transgenic mice and so the injection procedure was repeated with the unaltered YAC 35D8 in case the lack of expression was due to a frame shift, which may also have inactivated the 5-HTT gene. Three transgenic lines (A145, A145.1 and A145.2) were produced (Figure 2.3.1.2). Since the levels of expression of the 5-HTT did not obviously differ from those in lines expressing the modified construct, due to space and time constraints the modified YAC lines were used rather than breeding the new lines.

2.3.2 X-gal staining for Histochemical detection of β-galactosidase activity

Staining for *lacZ* expression was unsuccessful. At embryonic day 12.5 there was no staining at all. Embryos from day 13.5 showed light staining in the choroid plexus and possibly very lightly in the midbrain. This increased in 14.5- and 15.5-day embryos to include vertebral and toothgerm staining. An apparent pathway from the midbrain to the frontal cortex was also visible (Figure 2.3.2.1).

DNA samples were taken by removing one limb from the embryo and extracting the DNA as described for tailtip samples.

Analysis of Tail Tip DNA for Transgenic containing 35D8/D6 YAC

Founder	Sex	Colour	A	B	C	D	E	F	G	H	TATTOO No.
14	F	Ag	+?	+	-	-	-	-	-	-	A101.3
37	F	Ag	+?	+?	+	-	-	-	-	-	A102.4
60	M	Bl	+	+	+	-	-	-	-	+?	A103.1
89	M	Bl	+	+	+	+	+?	-	+?	+	A105.2
53	F	Ag	+	+	+	-	+	+	+?	-	A103
70	M	Ag	+	+	+	+	+	+	+?	+	A104
77	F	Ag	+	+	+	+	+	+	+?	+	A104.1
21	M	Ag	-	+	+	+	+	+	-	+	A102
28	M	Ag	+	+	+	+	+	-	+	+	A102.2
83	M	Ag	+	+	+	+	+	+	+	+	A105
92	?	?	+	+	+	+	+	+	-	+	Dead
8	M	Ag	+	+	+	+	+	+?	+	+	A101.2
36	F	Ag	+	+	+	+	+	+	+	+	A102.3
46	M	Ag	+	+	+	+	+	+	+	+	A102.5
85	F	Bl	+	+?	+	+	+	+	+	+	A105.1
26	M	Bl	-	-	+?	-	+	+	-	+	A102.1
24	M	Bl	-	-	-	-	-	+?	-	-	Killed
6	M	Ag	+	-	+?	-	-	+	+?	-	A101
7	M	Ag	+	+	+?	-	-	+	+	-	A101.1

Table 2.3.1.1 PCR analysis of mice generated by microinjection containing the 5-HTT gene
A-H = primer pair used as listed in table 2.2.2.1, Ag= agouti, Bl = black

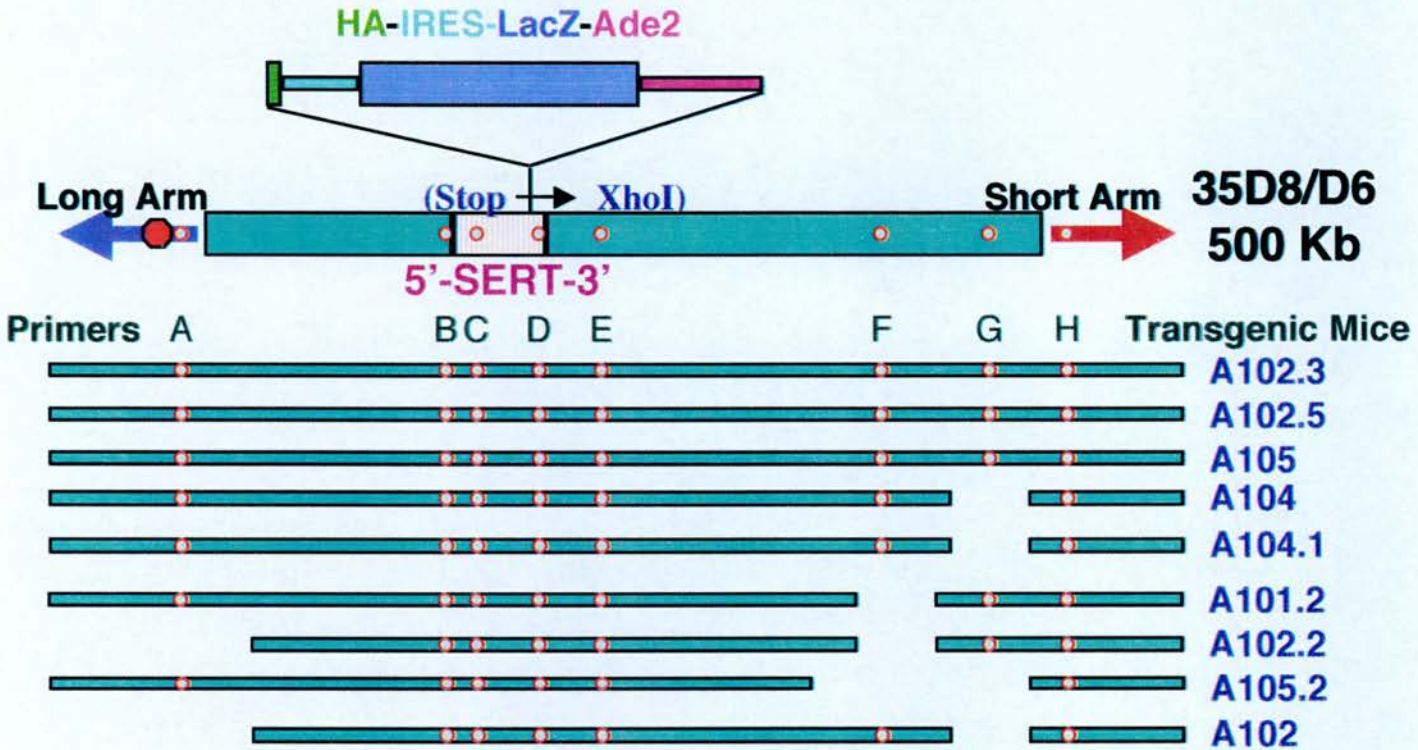

Figure 2.3.1.1 Transgenic mice expressing the h5-HTT gene. Comparison of integration patterns.

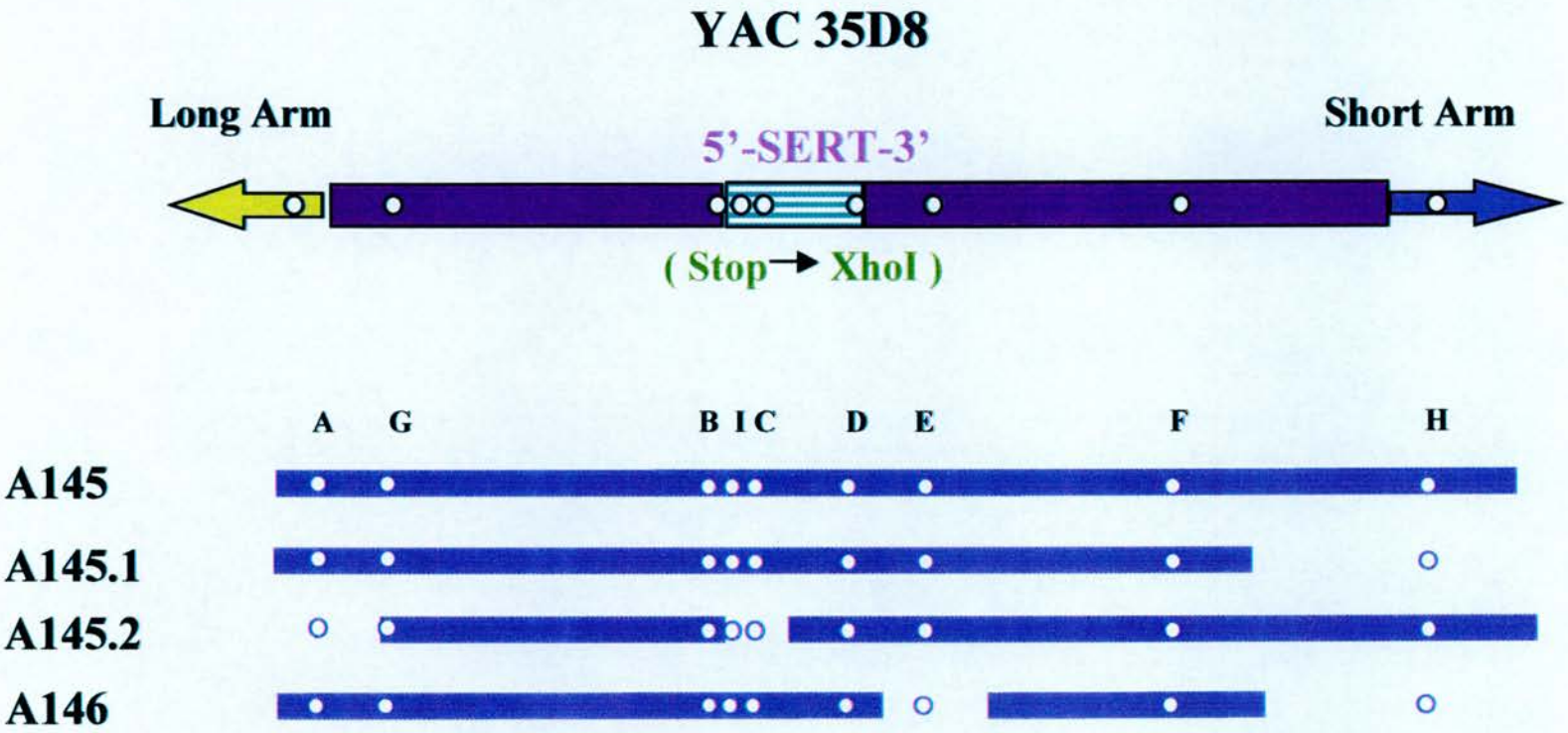

Figure 2.3.1.2 transgenic mice generated from the unmodified YAC 35D*. Integration sites.
A-H = primers as listed in to table 2.1 Primers: A= LongArm/Hygomycin B=1A promoter
C=SERT1B D=3'UTR E=D175294 F=D1S1594 G=D1S2009 H=Short arm I= GAP region

Figure 2.3.2.1 X-gal staining for β-galactosidase activity in Transgenic Mouse line A101.2 E14.5. A non-specific pathway from the midbrain to the cortex is visible

The staining pattern was then compared to the transgenic status of the animals (Table 2.3.2.1). X-gal staining did not relate to genetic status and so is non-specific. In adult transgenic brain slices there was no X-gal staining in any region, except background staining in the ventricles. There was also endogenous staining in the gut.

2.3.3 Immunohistochemistry for β-galactosidase, 5-HT and Haemagglutin-tag in adult brains

β-galactosidase antibody - There was no staining in any area after 24 hrs. of incubation with the fluorescence medium at either concentration of 1° antibody. The slides were therefore left for 24 hrs. and re-examined. There was still no visible fluorescence.

HA tag- there was no fluorescence after 24hrs. After another 48 hours very faint fluorescence was seen in the brainstem of transgenic mice. However this was very diffuse and negligible and so was probably due to background staining as it was also visible when binding was inhibited by co-incubation with the peptide against which the antibody was raised.

5-HT - After 24hrs. of development there was some fluorescence in the brains of transgenic and wild-type mice at both concentrations of 1° antibody. 5-HT projections are seen in the cortex and lateral ventricle of controls and transgenics and possible fluorescence in the ventricles of transgenic but not wild-type mice. The hindbrain of wild-type mice showed some staining in the dorsal raphé area in a pattern similar to that seen in other work (Bengel et al., 1997) (data not shown).

2.4 Discussion

The use of YACs to integrate a human gene into the mouse genome provides a method by which large amounts of DNA sequence, including most of a gene's long-range transcriptional control elements can be incorporated. Here the SLC6A4 and its

Line	No	age	brain +/-/d	choro id	tooth germ	face	spinal cord	verte brae	sex organ	tail tip	limb/ digit	kidney	jaw	PC R
A105.2	1	18.5	d	+	+	+	-	-	-	-	-	+	+	-
	2		?d	+	+	+	-	∂+		-	-	+	-+	-
	3		+	+	+	+	++	+		-	-	+	+	-
	4		?d	+	+	+	∂?	-		-	-	+	+	-
	5		?d		+	+	∂?	+		-	-	+	+	-
	6		?+	+	+	+	-	-	-	-	-	+	-	-
	7		+	+	+	+	-	++		-		+	faint	-
	8		?	+	+	+	-	-		-	-	+	+	+
	9		not	good	cut	+	-	++				+	+	-
	10		"	"	"	+	-	++				-	+	-
	11		-	-?	+	+	-	-				+	+	-
	12		-	-	-	+	-	-				-	-	+
	13		-	-	-	+	-	-				-	-	-
	14		+	+	+	+	-	+				+	+	+
Line	No	age	brain +/-/d	choro id	tooth germ	face	spinal cord	verte brae	sex organ	tail tip	limb/ digit	kidney spleen	jaw	
A101.2	1	13.5												-
	2													-
	3		?	+	-	-	-	-	-	-	?		-	+
	4		?	+	-	-	-	-	-	-	-	-	-	-
	5		-	+	-	-	-	-	-	-	-	-	-	-
	6		?	+	-	-	-	-	-	-	-	-	-	-
	7		?	+	-	-	-	-	-	-	-	-	-	-
	8		+	+	-	-	-	-	-	-	-	-	-	-
	9		+?	+	-	-	-	-	-	-	-	-	-	-
	10		+?	+	-	-	-	-	-	-	-	-	-	-
	11		-	?	-	-	-	-	-	-	-	-	-	+
	12		-	-	-	-	-	-	-	-	-	-	-	-
	13		+	?	-	-	-	-	-	-	-	-	-	-
Line	No	age	brain +/-/d	choro id	tooth germ	face	spinal cord	verte brae	sex organ	tail tip	limb/ digit	kidney spleen	jaw	
A102.5	1	14.5	+	+	+		?	?	+	+	-?			-
	2		+	+	+		+	-	-	-	+			-
	3		-	+	-		-	-	+	+	-			-
	4		+	+	-		-	?	?	-	?			+
	5		-	+?	-		-	-	-	-	-			-
	6		+	+	+		-	-	-	-	-	+		+
	7		+	+	-		-	-	-	-	-			+
	8		-	+	-		-	-	-	-	-			-
	9		?	+	-		-	-	-	-	-			-
	10		d	+	-		-	-	-	-	-			-
	11		-	+	-		-	-	-	-	-			-
	12		-	+	-		-	-	-	-	-			+

Table 2.3.2.1 Results of X-gal staining in embryos from h5-HTT mice.
Key: (+) staining is present, (-) staining is not present, (∂) staining is faint, (d) staining is diffuse, (?) staining is uncertain, Genetic status was defined by PCR analysis.

upstream and downstream regulatory sequence have been introduced to a mouse. The presence of the gene is identified by the PCR results with gene specific primers and nearby STS markers.

The use of YAC transgenics minimises many of the problems of transgenic technology as the large size of a DNA sequence maintains the expression patterns to the correct areas and prevents ectopic expression of the gene. However the large size also means that shearing of the chromosome is more likely to occur during microinjection. This can lead to breakage of the YAC and multiple incorporation sites or to the inactivation of the gene even though all the DNA may be present. Many of the founder mice have parts of the YAC missing. This is due to incorporation of some of the sheared parts of the YAC - probably into different sites in the genome- and loss of some bits of the YAC. Those mice where the markers for all the 5-HTT sites and the STS markers on either side are present were considered likely to contain the complete h5-HTT gene. The insertion of DNA into the pronucleus before cell division also ensures that the transgene is expressed in all cells rather than in a mosaic pattern. However, the insertion of such a large piece of DNA into the genome can also have unwanted effects – accidental KO where the transgene inserts into the middle of another gene effectively knocking it out. For example, Cases *et al* (1995) reported a line of transgenic mice in which integration of an interferon transgene caused a deletion in the gene encoding monoamine oxidase A (MAO-A), providing an animal model of MAO-A deficiency rather than one for overexpression of interferon. Placement of the transgene within the genome may also affect its expression and regulation; thus, wherever possible, several lines of transgenic animals should be studied to confirm that any alterations seen are due specifically to the transgene.

The copy number of inserted transgenes is lower with YAC than BACs as the DNA fragment is much larger (Giraldo and Montoliu, 2001). Normally multiple copies of transgenes are inserted end to end at the point of entry. In general, copy number directly relates to expression level and so different lines can show different phenotypes.

It was hoped that the incorporation of the HA-IRES-*lacZ* -ADE2 cassette would provide an efficient method to analyse the pattern of h5-HTT expression. However staining for *lacZ* expression was unsuccessful. There was light staining in embryos after 13 days which is the expected time of 5-HTT expression in tissues which might be expected to show the 5-HTT gene (Bruning et al., 1997; Schroeter and Blakely, 1996; Zhou et al., 2000). This proved to be non specific and X-gal staining was not seen in adult brains. It was possible that this was due to a failure at the level of incorporation of the *lacZ* reporter gene into the construct. A single base mis-incorporation or deletion can cause a frame shift and non-expression. This may have occurred, in this construct, when the HA sequence was inserted at the stop codon of the 5-HTT gene from the previous construct. A second possibility is that β-galactosidase expression was low, insufficient to detect over the background staining that is always expected in this type of experiment. β-galactosidase needs to form a tetramer for enzymatic activity diffuse low expression may not be sufficient for this to occur (Hirata et al., 1984; Jacobson et al., 1994; Juers et al., 2000; Kaneshiro et al., 1975; Pisani et al., 1990).

To answer this question immunofluorescence, which is more sensitive than chemical staining, was used to identify β-galactosidase expression and expression of h5-HTT protein with the HA tag in adult mouse brain sections. However, this was also unsuccessful under any concentration of 1° or 2° antibodies used. The same antibodies had previously been used successfully in cell lines in the laboratory. However, the HA tag was not easily detectable in mice expressing the VIP2 receptor clone from which the HA-IRES- *lacZ* -ADE2 cassette was taken. The levels of expression may be too low to detect over a background of nonspecific staining that will be present in any such experiment, or the antibody may be inefficient in animal tissues. An antibody against 5-HT in parallel experiments identified projections in both wild-type and transgenic mice in the cortex and brainstem. These areas are consistent with the pattern of 5-HT innervation and with the expression pattern of 5-HTT RNA in other mouse studies (Bengel et al., 1997). This staining in the expected areas shows that 5-HT neurons are being expressed in these animals and

confirms that the secondary antibody and technique are functional. This leads to the conclusion that there was a problem with the incorporation of the marker sequence into the YAC resulting in loss of activity in the *lacZ* gene and HA tag expression. There have been several reports of problems with *lacZ* expression in transgenic animals although the reason is often unknown.

This chapter reports the generation of transgenic mice containing the h5-HTT gene as identified by PCR. However, as neither the β-galactosidase nor HA markers were available in these animals in order to study the expression of the human gene in these animals, two alternative, more traditional, strategies were used. Firstly the RNA was analysed by RT-PCR , RNase protection analysis , and *in situ* hybridisation to show expression and location of the human RNA (Chapter 3). Secondly to show protein expression [^{3}H]citalopram binding assays and auto-radiography (Chapter 4) were used.

Chapter 3

Localization and quantification of RNA expression in the brain of h5-HTT transgenic mice

3.1 Introduction

3.1.1 5-HTT RNA expression in adult mice

In adult mice 5-HTT RNA is expressed almost exclusively in the midbrain and hindbrain. It is expressed throughout the mesencephalic raphé nuclei (Bengel et al., 1997; Schroeter and Blakely, 1996) and is highest in the dorsal and median raphé nuclei (Bengel et al., 1997). *In situ* hybridisation and northern hybridization analyses (Bengel et al., 1997; Blakely et al., 1991; Hansson et al., 1998; Hoffman et al., 1991; Lesch et al., 1993) do not identify 5-HTT mRNA in any other region of the adult mouse or rat brain except a very low and diffuse expression in the dentate gyrus of adult rat hippocampus (Hansson et al., 1998). Using the more sensitive RT-PCR technique low levels of expression of 5-HTT RNA were found in the frontal cortex and hippocampus of rats despite the lack of 5-HT cell bodies in these areas.(Lesch et al., 1993). This has not been repeated and has not been studied in mice.

3.1.2 RNA expression during development

5-HT has many roles in development (see introduction) (Lauder et al., 1983; Whitaker-Azmitia, 1991; Whitaker-Azmitia et al., 1996). In the brain specific 5-HT neurons are first seen at E13 in the rat (Lauder, 1990; Wallace and Lauder, 1983) but 5-HTT RNA is seen, well before the development of synapses, at E10 in the mouse (Schroeter and Blakely, 1996) and E11 in the rat (Hansson et al., 1998).

Most of the work on 5-HTT RNA localization during development has been done in rats. However binding studies using 5-HTT specific ligands in mice show that the

overall spatiotemporal expression pattern of the serotonin transporter in the mouse concurs with the localization of serotonin in the rat embryo (Bruning et al., 1997), thus where evidence in mice is not available it is feasible to extrapolate from the data available in rats. In rats 5-HTT RNA is expressed both in cells which synthesise 5-HT and other cell types where serotonin probably has a less traditional role.(Hansson et al., 1999; Zhou et al., 2000). The hippocampus produces 5-HTT RNA from early embryonic through to postnatal development (Hansson et al., 1998) and the piriform cortex, olfactory nucleus and hypothalamus (Hansson et al., 1998) and the thalamus and neural crest-derived sensory neurons(Hansson et al., 1999) also show 5-HTT RNA activity during ontogeny.

3.1.3 RNA expression patterns and the study of gene expression.

In modified animals which contain higher levels of h5-HTT DNA the RNA may be being expressed in tissues which do not normally express 5-HTT or in greater, more easily detected, concentrations in areas with normally low expression. Analysis of RNA expression was used to determine gene expression and localization as the presence of the marker gene *lacZ*, which would have been used to determine areas where the transgene was being expressed, was not detectable. RT-PCR provides a very sensitive method of RNA detection but was difficult to quantify and without detailed dissection does not provide more than general localization. RNase protection assays were therefore also used for quantification. *In situ* hybridization provides a sensitive method of RNA localization in tissue sections with a high level of specificity for either the human or mouse form. This combination of techniques provides a comprehensive picture of 5-HTT gene expression in these animals.

3.2 Methods of RNA analysis

3.2.1. Extraction of RNA from tissue samples

Heterozygote h5-HTT mice were killed by cervical dislocation and the brain removed and dissected immediately. For most studies the areas taken were: the

cortex to study possible differences between transgenic and wild-type mice, brainstem/midbrain containing the raphé nuclei, and the cerebellum as a negative control. A coronal cut was made immediately anterior to the cerebellum and followed by the separation of the cerebellum from the brainstem. The midbrain was dissected and pooled with the brainstem sample. A sagittal cut was made down the midline between the two hemispheres of the cerebral cortex. The cortex was separated from the hippocampus and striatum and the remaining structures were discarded. The two halves of the cortex were then pooled. Samples were frozen on dry ice for later assay or homogenised immediately in RNAzol B (Biogenesis Ltd. England) using glass homogenisers and extracted according to the manufacturers instructions. Samples were then precipitated with isopropanol and resuspended in tissue culture grade water (Sigma) and stored at –70 °C. under ethanol until required. JAR cells -a human choriocarcinoma cell line expressing the 5-HTT (Cool et al., 1991)- were used to provide human RNA. RNA was extracted by homogenising the cells in 2ml of RNAzol B per 10^{-6} cells and isolated according to the manufacturer's instructions. Before use RNA was quantified using OD 260/280 ratios of the samples measured on a spectrophotometer. RNA was considered pure if the 260/280 ratio was 1.9-2.1.

3.2.2 RT-PCR

cDNA synthesis

RNA samples were transcribed using Omniscript kits (Qiagen) according to the manufacturer's instructions.

1µg samples of total RNA from different brain regions were transcribed at 37°C. for 1 hour in the presence of RNAse inhibitors. In most experiments the whole cortex, brainstem and midbrain and cerebellum were used in order to provide sufficient RNA. In experiments for closer localization in the wild-type samples were taken from the frontal cortex, basal ganglia, midbrain, brainstem, hypothalamus,

hippocampus, and cerebellum for analysis. To facilitate comparison the same dissection was carried out as used when analysing neurochemistry (see section 5.2)

cDNA amplification

For the expression analysis of human 5-HTT half the resulting cDNA was amplified using ^{32}P end-labelled primers E4 (CTA CCT CAT CTC CTC CTT CAC g (Tm 62.1 oC)) and E7 (TTg TTg TTg AAC TTg TTg TAg C (Tm 54.7 °C)) from the exons 8-11 region. This pair of primers was selected as the area amplified is of a similar size in the two species (human 510 bp, mouse 499bp) but contains several differences in restriction enzyme sites between the two species (see Figure 3.2.2.1).

Primers were labelled by PNK phosphate substitution. 30pmols of each primer were incubated for 30min at 37°C in the presence of 90 μCi, 30pmols of γ[32P]ATP (Amersham Pharmacia Biotech, UK). Primers were then precipitated in the presence of 20μg yeast total RNA as a carrier, washed to remove excess label, and resuspended in tissue-culture-grade water (Sigma). Primers were made up to sufficient concentration with cold primer and used in PCR reactions.

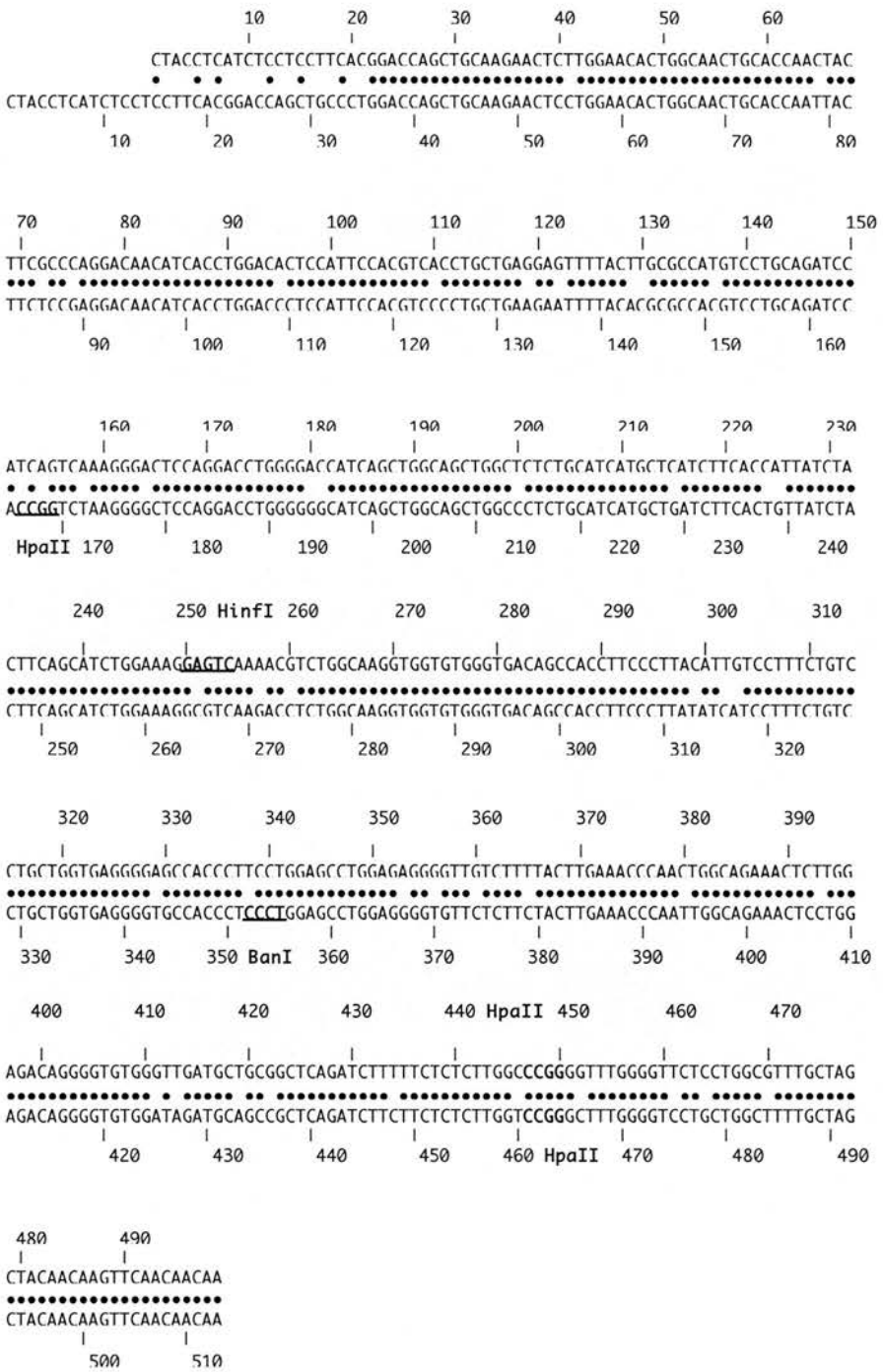

Figure 3.2.2.1. Comparison of Mouse (top) and Human (bottom) cDNA sequences as generated by RT-PCR with primers E4 and E7 Showing restriction endonuclease sites.

Analysis of cDNA products

To study the expression of human or mouse RNA the cDNA products were then cut by restriction endonuclease digestion. The enzymes used and the products created are described in Table 3.2.2.1.

Restriction enzyme	Species cut	Product Size
BanI	h	341, 172 bp
HinfI	m	249,163,87 bp
HpaII	h	295,166,52bp
	m	447,52 bp

Table 3.2.2.1 Restriction endonucleases used for analysis of RT-PCR and the fragments generated. (h)= human (m)= mouse

The digested samples were then separated on a 5% non-denaturing acrylamide gel at 80-100 volts until the dye front (65bp) ran off., dried and exposed to Kodak biomax film with a intensifying screen for at least 48 hrs. or to a phosphoimager cassette.

For Wild-type localization studies PCR with unlabelled E4, E7 primers only followed the cDNA transcription.

RT-PCR reactions were carried out using a HYBAID Omni-E PCR machine with Hotlid. PCR reactions were carried out on a HYBAID Omni-gene PCR machine. Phosphoimaging used a Cyclone phosphoimager storage system with Optiquant image analysis software (Hewlett Packard,UK). Primers were obtained from MWG Biotech. (Germany) and Taq Polymerase ,and other reagents from Promega. Image analysis was done using NIH image 1.62 after scanning using a EPSON II scanner. Bands were selected visually and the area further defined by intensity greater than 5% over background. Signal density was measured in OD units converted to $\mu Ci.\mu g^{-1}$ from standards.

3.2.3 RNase Protection assay

RNase protection assays are a form of solution hybridisation using a riboprobe complementary to the sequence of interest and the properties of RNase T and RNase A1 that digest single stranded RNA and mismatched strands. This allows the identification of several similar products with the same probe and within the same experiment.

A cRNA riboprobe containing a 375bp complementary region from exons 8-11 of the human 5-HTT gene (Figure 3.2.3.1) was generated by synthesising a fragment of the human cDNA using RT-PCR and inserting this into a T4 vector. This was linearised using NcoI and transcribed using SP6 enzyme in the presence of excess ^{32}P labelled CTP. The labelled product was purified through a G-50 column (Boerrhinger- Mannenheim), precipitated and 1µl counted on a scintillation counter. $1x10^5$ dpm of labelled probe per sample was added to a 20µg pellet of sample RNA. Samples were assayed using the RPAII kit (Ambion) according to the manufacturer's instructions. Briefly, pellets of freshly precipitated and dried tRNA were incubated with the labelled cRNA probe overnight at 42 °C to allow hybridisation to occur. After hybridisation RNase A/T1 was used to digest any single stranded RNA, the area hybridised to the probe was protected and generated a full-length human 375bp product. The human probe hybridised to the mouse RNA where there was a 4bp area of mismatch (Figure 3.2.3.1). This section was also digested by the Rnase, generating two smaller RNA fragments of 274 and 85 bp. Products were separated on a 7.5% denaturing acrylamide gel. The gel was dried and exposed to Kodak biomax film in the presence of an intensifying screen for at least 1 week or to a phosphoimager cassette.

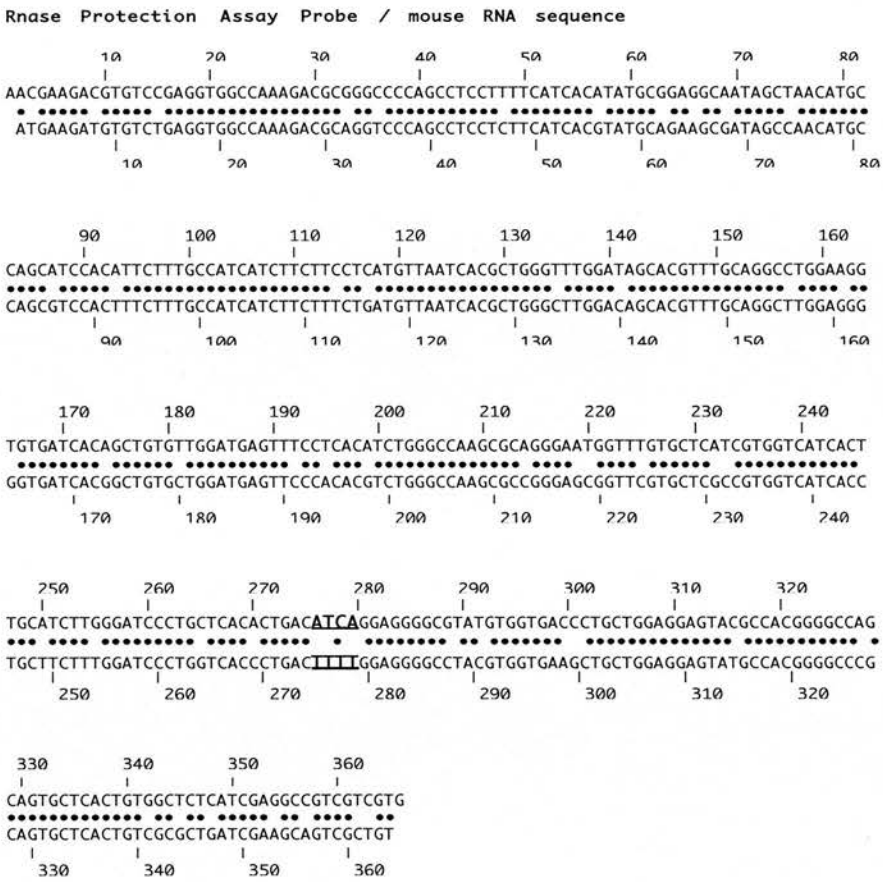

Figure 3.2.3.1 comparison of the ribobrobe from the human sequence and mouse RNA. Sites of sufficient mismatch for RNase digestion are highlighted.

3.2.4 **In situ *hybridisation analysis of RNA expression and localisation***

Preparation of brain sections

Six male wild-type C57Bl6/CBA animals (Charles River) and six male +/- heterozygous h5-HTT transgenic mice (3-6 months old), on a C57Bl6/CBA background, were killed by cervical dislocation. Brains were removed as quickly as possible; about 7 minutes per mouse, and frozen in an isopentane bath at -30 to -35°C. for a few minutes until bubbling ceased then transferred to dry ice and stored at -70°C. until required.

12 micron coronal sections were cut at -20°C. in a cryostat, according to levels in the mouse brain atlas by Franklin & Paxinos (Franklin, 1997). The levels for coronal sections used, according to the atlas, are listed in brackets. Frontal cortex (8-12), striatum (16-20), hippocampus/SNR (26-30), anterior raphé (35) and posterior raphé (36-38) and thaw mounted on gelatinised slides. Once mounted, sections were maintained at –20°C then stored at –70°C until required.

For *in situ* hybridization, slides were pre-treated as follows. Sections were fixed in 4% paraformaldehyde, acetylated for 10 minutes in 0.1M triethanolamine HCl pH 8, then dehydrated in increasing concentrations of ethanol and air dried. Sections were then stored overnight at –20°C.

Hybridisation

Two cDNA probes were used: (1) a probe for the human transporter (obtained from Qi Pei, Department of Pharmacology Oxford University) which could hybridise with the mouse transporter under low stringency conditions; and (2) a specific 3' mouse probe selected from the sequence in the data base for low homology with the human transporter sequence (obtained from Sigma Genosys Ltd.). The probe information is listed in table 3.2.4.1

Species	Length	Sequence	% GC	% mismatch
human	42bp	CACCCAgCAgATCCTCCAgAA CCACCCCgggCTgAAgCCgAg	66	9.5%
mouse	40 bp	CgTgTCACCAgCTAATgTggCA gTAACTCCAAgAgAgTTC	42.5	0%

Table 3.2.4.1 Human and mouse probe sequences % mismatch is that compared to the mouse sequence in experiments where both human and mouse were detected using the same probe.

The probes were tested in a preliminary experiment for species specificity and in the presence of the antisense or cold probe. There was no cross hybridisation under the conditions used.

Probes were end-labelled with ^{35}S (NEN Life Science Products 12.5mCi/ml; specific activity 1250Ci/mmol) at 6µl per 6pmol probe and incubated at 37 °C for 30 minutes with TdT enzyme (Promega). The reaction was stopped and the labelled probe was purified through Sepadex G-50 gel. The purified probe was counted on a beta counter and diluted to a concentration of $1.2x10^6$ cpm/100µl in hybridisation buffer containing for most of the experiments 50% formamide, 4x SSC buffer with 0.05mM DTT (see table 3.2.4.2 for solution compositions). 100 µl of solution was used per section. Slides were covered with glass cover slips and then placed in lidded incubation trays containing filter paper soaked in 50ml of 50% formamide with 4xSSC to prevent drying out. Sections were then hybridised over night in a sealed oven at the appropriate temperature.

Calculation of hybridisation temperature:

The hybridisation of a riboprobe to sample RNA is controlled by the melting temperature of the probe. The ideal temperature for hybridisation is one sufficiently high to maintain specificity but not so high that the probe becomes damaged.

Melting temperature is influenced by 1) length of probe 2) base composition 3) extent of sequence homology 4) composition of hybridisation and wash solution. The formula below describes the interaction of these factors (Davis et al., 1986):

$$T_m = 16.6log10\ (molarity\ of\ monovalent\ cations) + 0.41\ (\%GC\ content\ of\ probe) + 81.5 - (\%\ of\ mismatches) - (675/length\ of\ probe\ in\ bases) - 0.65(\%formamide\ in\ solution)$$

Incubation and washing are performed at temperature = T_m-15 °C.

Using this formula and the information in Table 3.2.4.1, conditions were chosen to provide the optimum temperature by altering the molarity of monovalent cations in the hybridisation solution. Table 3.2.4.2 describes the conditions used to detect human and mouse RNA.

Probe	Species detected	% formamide	SSC	Hybridisation temperature (°C)
human	Human only	50%	2x	37
human	Human/mouse	50%	4x	33
mouse	Mouse	50%	4x	34

Table 3.2.4.2 Composition of hybridisation buffer and conditions used for the detection of human and mouse RNA in mouse brain sections. SSC =(sodium chloride / sodium citrate buffer - composition see appendix 1)

After hybridisation coverslips were removed in 1xSSC. Slides were the washed three times in hot SSC (See Table 3.2.4.3 for conditions) for 20 minutes and twice at room temperature for 1hour. All solutions were DEPC treated.

Wash conditions are also dependent upon the formula above. During washing there is no formamide present in order to facilitate the removal of non-specific bound probe. The higher the concentration of SSC solution in the buffer the higher the concentration of monovalent-cations. At higher concentrations of cations the bonds formed between RNA are stronger and so decreasing the concentration of SSC increases the specificity. Conditions used are listed in Table 3.2.4.3.

Probe	Species detected	% Formamide	SSC	Hot-wash temperature (°C)
human	Human only	0%	0.25x	55
human	Human/mouse	0%	1x	55
mouse	Mouse	0%	1x	55

Table 3.2.4.3 Composition of washing solution and conditions used.

Slides were rinsed in water to remove any salts and dried before being exposed to Betamax film for 4 weeks. Image analysis was by computer-based image analysis program (MCID/M4, Imaging Research Inc) and Microsoft Excel.

After background subtraction, optical density (OD) values were converted to $\mu Ci.g^{-1}$ tissue equivalent using standard curves of OD versus radioactivity in $\mu Ci.g^{-1}$ from standards exposed to the same film. OD measurements were made by selecting a suitable measuring tool, or box, of appropriate dimension, overlaying the structure of interest, and measuring the optical density of that area. Measurements were made bilaterally on 2-three sections for each mouse and each set of conditions. Significance was defined as $p<0.05$ in a student t-test after F-test for analysis of variance between mice in each group

3.3 Results

3.3.1. Tissue localisation of human and mouse RNA

3.3.1.1 Tissue localisation in wild-type animals

RT-PCR analysis of tissue samples shows that wild-type animals express 5-HTT in the midbrain and brainstem regions that, in this dissection, contain the raphé nuclei. There was no detectable expression in the cortex, hippocampus, hypothalamus, basal ganglia or cerebellum (Figure 3.3.1.1). In none of the RT-PCR or solution hybridisation experiments was RNA detected in any part of the brain of wild-type

Figure 3.3.1.1 RT-PCR detection of 5-HTT RNA expression in tissue samples from the brain of wildtype mice. A) hippocampus, B) frontal cortex, C) brainstem, D) hypothalamus, E) cerebellum, F) midbrain, G) basal ganglia, H) JAR cells 0.5µg ,I) JAR cells 1µg

mice, except the midbrain and brainstem areas. In the dissection used the substantia nigra and globus pallidus, which have been shown to express 5-HTT RNA in mice, were not assayed separately.

3.3.1.2 Tissue expression in transgenics

All transgenic animals show 5-HTT expression in the brainstem region, which here includes both midbrain and brainstem raphé. When the cDNA product was digested with BanI endonuclease (Figure 3.3.1.2), which cuts human DNA, transgenic samples show the product of smaller bands at 342 and 172 bp which is consistent with the expression of the human 5-HTT in lines A102.3, A102.5, A104 and A145.1 but not A102.2. Not all of the DNA was digested, showing the existence of endogenous 5-HTT. When HinfI endonuclease, which digests only mouse DNA, is used, the expected bands are also seen in all lines (Figure 3.3.1.3). The endonuclease HpaII cuts both human and mouse DNA with different size products. Human 295,166 and 52bp and mouse 447 and 52 bp. When the cDNA is digested with this enzyme, 5 bands rather than the expected 4 are seen even when the enzymatic digestion is allowed to continue for longer than necessary for complete digestion of DNA from wild-type animals (Figure 3.3.1.4). This is probably as a result of hybridisation occurring between human and mouse sense and antisense strains to produce a double stranded DNA that does not contain restriction endonuclease sites. Thus the enzymes which cut only a single species, BanI and HinfI, can not be used for quantification as the residual band may contain both hybrid and undigested DNA and so not provide a true measure of expression for one species.

Transgenic mice also show 5-HTT expression in the cortex that was not seen in wild-type mice. This was completely digested by BanI endonuclease showing that this is due to specific transgenic expression of the human RNA and not to up-regulated endogenous expression (Figure 3.3.1.5).

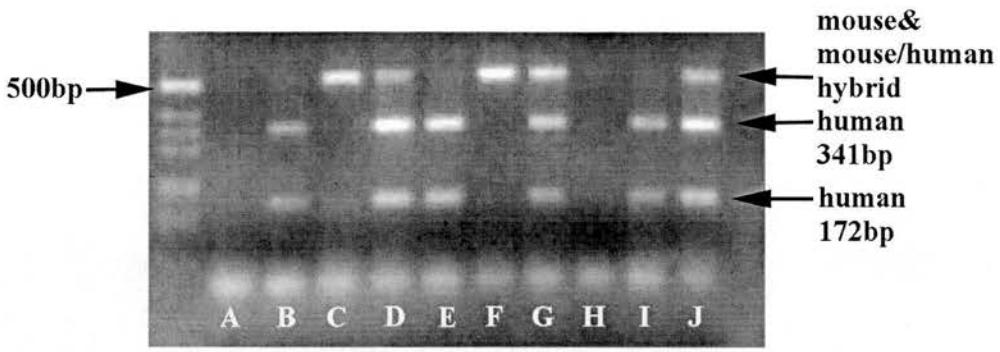

Figure 3.3.1.2 BanI digest of cDNA from cortex (A-E) and brainstem (F-J) of wildtype A,F; A102.3 B,G; A102.5 C,H; A145.1 D,I; A104 E,J

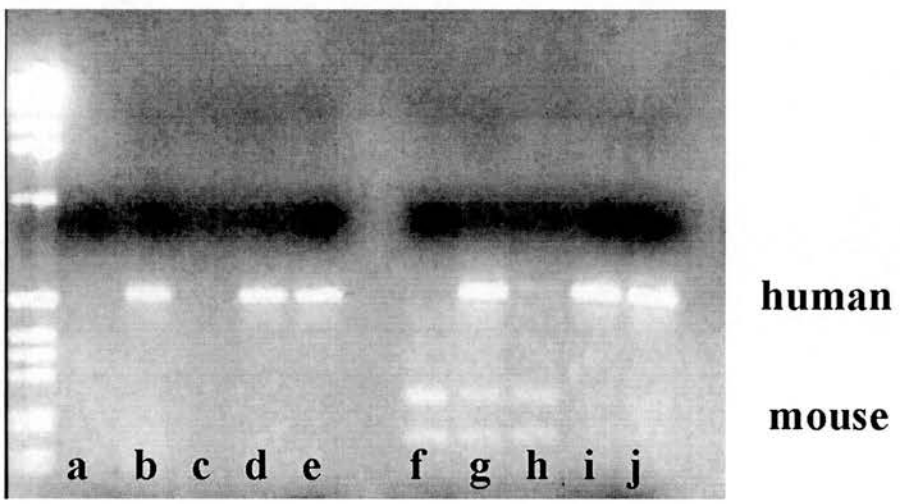

Figure 3.3.1.3 HinfI digest of cDNA from cortex (A-E) and brainstem (F-J) of wildtype A,F ;102.3 B,G ; 102.5 C,H ;145.1 D,I ;104 E,J

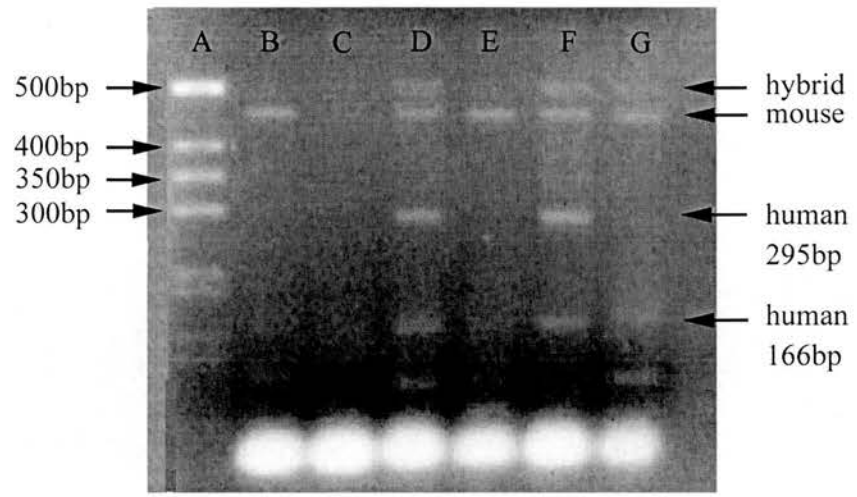

Figure 3.3.1.4 HpaII digest of cDNA from Brainstem of: B=wildtype, C=A102.2, D= A102.5, F= A145.1, G=A104

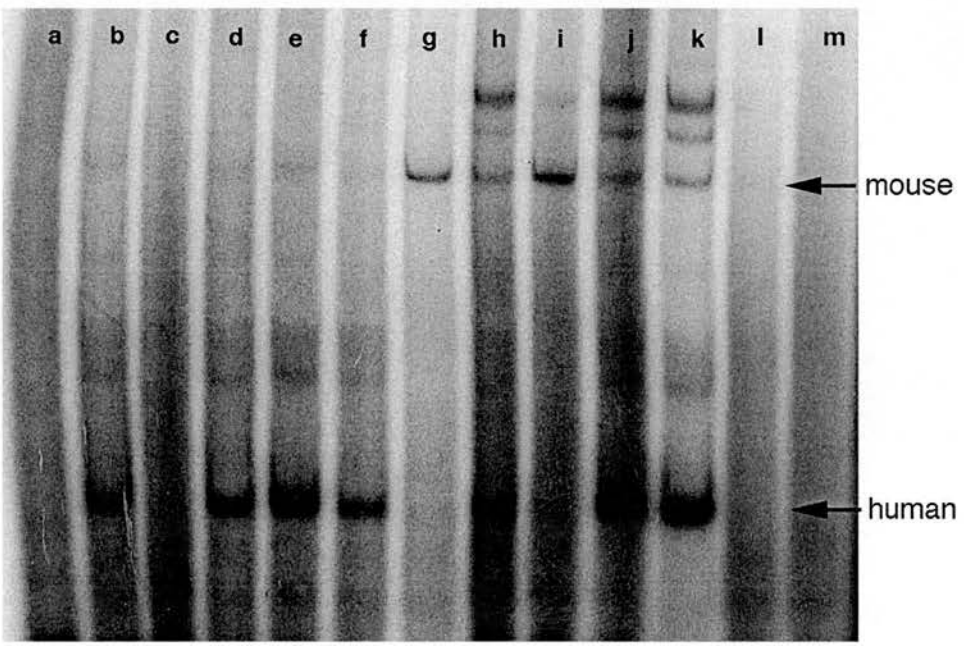

Figure 3.3.1.5 HpaII digest of RT-PCR products from a) WT cortex, b) 102.3 cortex, c) 102.5 cortex, d) 145.1cortex, e) 104 cortex, f) 102.2 cortex, g) wt brainstem, h) 102.3 brainstem, i) 102.5 brainstem, j) 145.1 brainstem, k) 104 brainstem, l) 102.2 brainstem, m) wt cerebellum

All the lines tested show this cortical expression. However it is not consistently present in every sample. This is most probably due to individual sample degradation as repeat experiments with the same RNA sample detected 5-HTT expression.

The cerebellum and basal ganglia of wild-type mice do not show 5-HTT RNA expression (consistent with previous work: Bengel et al., 1997). The basal ganglia of line 102.3 also do not show 5-HTT expression of either human or mouse gene. The cerebellum of some transgenic lines shows slight 5-HTT RNA expression. Lines 102.2 and 102.3 have the highest levels whilst 102.5, 145.1 and 104 do not appear to have 5-HTT RNA in these areas. There is very little 5-HTT expression in the cerebellum of primates or rodents.

3.3.1.3 Summary of RT-PCR results

Thus RT-PCR detects the presence of human 5-HTT expression in the cortex of most transgenic mouse lines, though this is much smaller in A102.2, and confirms that endogenous mouse 5-HTT is not detectable in the cortex of wild-type animals.

Transgenic animals also show expression of both human and mouse 5-HTT in brainstem tissues.

3.3.1.4 Proportion of h5-HTT expression.

Analysis of RT PCR results using Kodak image analysis software to measure proportional luminosity of ethidium bromide staining shows considerable variation in expression between wild-type and transgenic mice (a 1.4-6 fold increase), and also between the different transgenic lines, with 145.1 and 104 having the greatest increase and 102.2 the least. Cortical expression is also greatest in 145.1 with smaller amounts in 102.3 and 104, again with 102.2 the lowest of the expressing lines and 102.5 showing low expression in the cortex (Table 3.3.1.1). However the use of ethidium bromide as a stain is inaccurate because of the steep concentration-

	Cortex		Brainstem	
	Human	Mouse	Human	Mouse
Wildtype	N/A	NP	N/A	1
A102.3	5603	NP	5.8±0.07	1
A102.5	NP	NP	1.6	1
A102.2	5763	NP		1
A145.1	7053	NP	7.8±3.1	1
A104	NP	NP	9.3±1.6	1

Table 3.3.1.1 Human and Mouse RNA expression in the cortex and brainstem of wildtype and transgenic mice by RT-PCR with labelled primers.

N/A =not applicable NP = not present . Cortical amounts are absolute measurements of RNA expression-μCi.μg^{-1}. The brainstem measurements are given as the ratio of human to endogenous mouse in each line. N=3

luminosity curve. A comparison of products using radio-labeled probes is more accurate. For RT-PCR to be semi-quantitative an internal control consisting of a modified sample DNA sequence, which produces a smaller product to allow comparison of the products, is required. In the protocol described here the presence of the mouse form of the transporter provides an in-built control for the expression of human 5-HTT in the brainstem region.

Table 3.3.1.1 lists the concentration of human and mouse RNA in the brainstem of different lines of transgenic animals and in the wild-type. All the transgenic lines, except A102.2, express the human 5-HTT in the brainstem in addition to the mouse form the expression varies from 1-4 fold and is greatest in lines A102.3 and A145.1. In addition transgenic mice, with the exception of A102.5, express the human 5-HTT in the cortex.

3.3.2 RNase protection assays.

RNase protection assays are as sensitive as RT-PCR but easier to quantify, though they require larger amounts of sample RNA. RNase protection concurs with RT-

PCR that there is no 5-HTT mRNA expression in the cortex of wild-type mice. 5-HTT mRNA was expressed in the cortex of all the transgenic lines tested (except A102.5 and A104) and in the brainstem (Figure 3.3.2.1).

There were three products in the brainstem – coinciding with the size of the human and two mouse, protected fragments. The ratio of human to mouse RNA expression was 1-4 fold in the brainstem.

3.3.3 Localisation and expression of mouse 5-HTT RNA in wild-type and transgenic mice

In situ hybridisation was used to provide a more specific localization of the h5-HTT expression in transgenic line 102.3 and wild-type mice than that obtained by tissue analysis. Three sets of conditions were used: A mouse specific probe to detect mouse 5-HTT. A human specific probe at high stringency to detect only human 5-HTT RNA and finally the same human probe at low stringency where it also detected mouse 5-HTT to compare the total level of 5-HTT RNA expression.

Coronal sections of the wild-type mouse brain at various levels of the neural axis including the frontal cortex, hippocampus, striatum and raphé areas showed a distribution of 5-HTT RNA consistent with that typically seen for serotonergic neurons. 5-HTT RNA was found only in the mesencephalic raphé nuclei (Table 3.3.3.1). The highest signal was found in the dorsal raphé with less in the median raphé (Table 3.3.3.1) and DRVL (data not shown). This is consistent with the hybridization expression pattern seen in mice (Bengel et al., 1997). There was no detectable expression in the hippocampus or any other brain region studied.

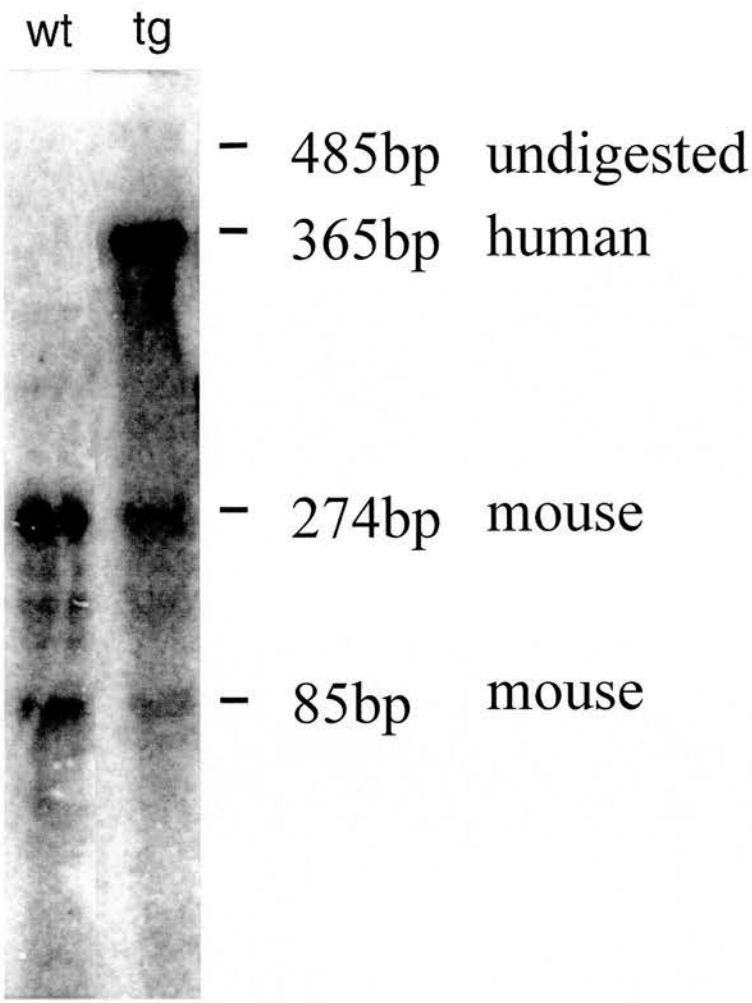

Figure 3.3.2.1 Representative RNAse protection assay using RNA from brainstem of wild type (wt) and transgenic line A102.3 (tg) mice.

	Raphé Nuclei (mouse probe)	
	Dorsal	**Median**
TgA102.3	1498±164	531±72
Wild-type	1498±359	572±125

Table 3.3.3.1 The expression of endogenous mouse 5-HTT RNA in the raphé nuclei of wild-type and transgenic mice. Data expressed as μCi/g tissue compared to standards n=6 ±SEM

There was no difference in mouse 5-HTT RNA levels in transgenic animals compared to wild-type as detected with the m5-HTT specific probe (Table 3.3.3.1).

The distribution of 5-HTT RNA was identical to that seen in wild-type mice and no expression was seen outside the raphé area. There was no change in level of expression in either the dorsal or median raphé. Thus the regulation of endogenous mouse 5-HTT is not affected by the inclusion of human 5-HTT promoter regions in the transgene and so any alterations in level or distribution of 5-HTT RNA expression detected with the human probe under low stringency conditions results from the production of h5-HTT RNA.

3.3.3.1 Expression of Human 5-HTT RNA in transgenic mice

The human 5-HTT riboprobe when used under low stringency conditions detects both human and mouse 5-HTT. This probe was also used under high stringency conditions (see Section 3.2.4) but under these conditions a higher level of damage to the sections occurred reducing the available measurements in the brainstem. Therefore the data presented are from experiments under low stringency conditions.

As mentioned above there is no alteration in mouse 5-HTT RNA in transgenic animals so any alterations seen with the human probe are because of human 5-HTT expression.

	Raphé Nuclei (Low stringency Human probe)		
	Dorsal	**median**	**DRVL**
Tg A102.3	2578±266**	938±164	1118±161 *
Wild-type	890±71	521±37	539±60
Ratio mouse:human	1:1.9	1:0.8	1:1

Table 3.3.3.2 The expression of 5-HTT RNA in the raphé nuclei of wild-type and transgenic mice *=$p<0.05$ **= $p<0.01$. Data expressed as μCi/g tissue compared to standards n=6 ±SEM

Sections used were adjacent to those used for the hybridisation with the mouse probe described above. In the brainstem area there is a significant increase in 5-HTT RNA levels in the raphé nuclei of transgenic mice (Figure 3.3.3.2, Table 3.3.3.2). The increase is 1.8 fold in the median raphé, 2 fold in the DRVL and 2.9 fold in the dorsal raphé. Thus for each copy of the endogenous mouse 5-HTT there are 0.8-1.9 copies of the human 5-HTT RNA.

3.3.3.2 Expression of 5-HTT RNA outside the raphé

In addition to increased expression in the raphé nuclei transgenic animals also show low-level expression in areas outside the brainstem area to which it is confined in wild-type animals.

Transgenic animals show h5-HTT hybridization in the hippocampus throughout CA1-CA3 and dentate gyrus (Figure 3.3.3.3, Table 3.3.3.3). This expression is at a much lower level between $1/12^{th}$ to $1/16^{th}$ of that seen in the raphé with the same probe. It is not possible from these results to identify a specific cell type that expresses the RNA. The same probe at high stringency conditions shows similar results and there is no significant difference between the two (Table 3.3.3.4).

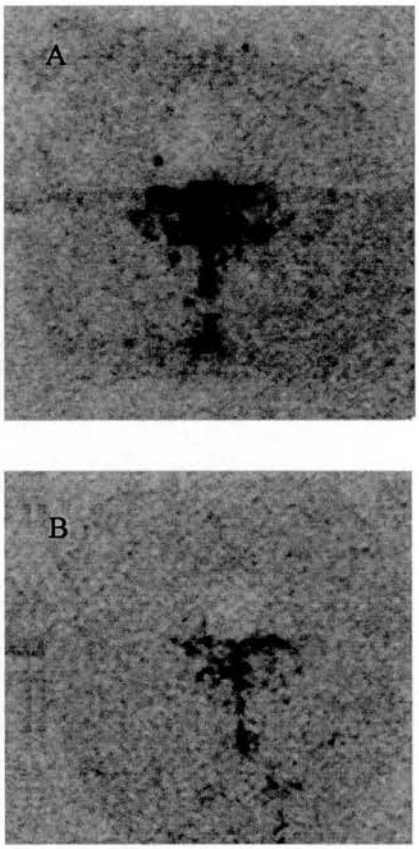

Figure 3.3.3.2 Localisation of 5-HTT RNA expression in the raphe nuclei of transgenic (A) and wildtype (B) mice. Detection with a human 5-HTT probe under low stringency conditions.

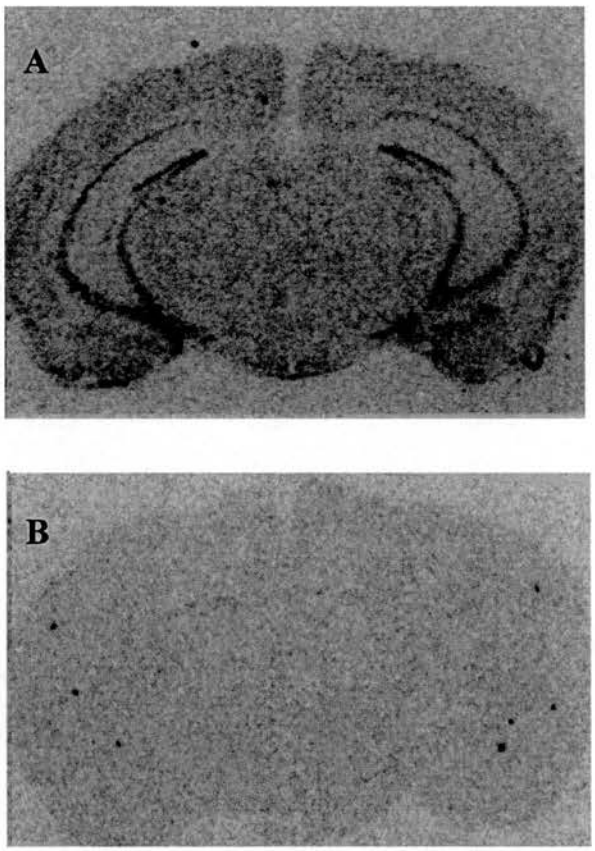

Figure 3.3.3.3 Localisation of h5-HTT RNA in the hippocampus of transgenic (A) and wild type mice (B). Detection is with a human 5-HTT probe under low stringency conditions.

	Hippocampus (Low stringency human probe)			
	CA1	**CA2**	**CA3**	**Dentate gyrus**
Tg A102.3	138±7	143±10	160±10	207±14
Wild type	ND	ND	ND	ND

ND = Not Detected

Table 3.3.3.3 Expression of 5-HTT in the hippocampus of transgenic mice. Data expressed as μCi/g tissue compared to standards n=6 ±SEM

	Hippocampus (High stringency human probe)			
	CA1	**CA2**	**CA3**	**Dentate gyrus**
Tg A102.3	166±9	182±7	205±13	278±12
Wild type	ND	ND	ND	ND

Table 3.3.3.4 Expression of human 5-HTT in the hippocampus of 5-HTT transgenic mice using a human specific probe at high stringency. Data expressed as μCi/g tissue compared to standards n=6 ±SEM

Further to this expression; low level 5-HTT RNA expression is also found in the piriform cortex and granular cell layer of the olfactory nucleus of transgenic animals (Figure 3.3.3.4). These areas do not show 5-HTT RNA expression in adult mice but do in embryos (Hansson et al., 1998).

3.4 Discussion

The mouse form of the 5-HTT mRNA was found throughout the brainstem raphé nucleus but not in the cortex or hippocampus in both control and transgenic animals. This is consistent with previous published studies (Bengel et al., 1997). 5-HTT mRNA, detected with a h5-HTT probe under high stringency conditions, was found in raphé; hippocampus CA1-CA3, and dentate gyrus; piriform cortex; and the

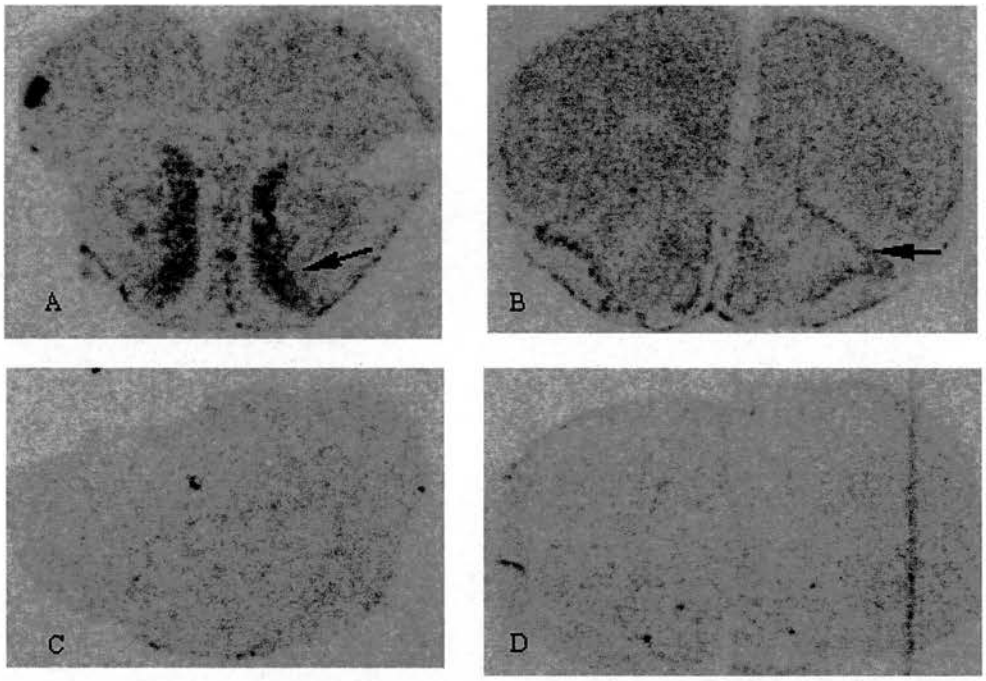

Figure 3.3.3.4 Localisation of h5-HTT expression in the granular layer of the olfactory nucleus (A) and piriform cortex (B) of transgenic mice and wildtype mice (C, D). Detection is with a human 5-HTT probe under low specificty.granular layer of the olfactory nucleus. The same probe under low stringency conditions found 5-HTT mRNA in the same regions.

There was no difference in level of m5-HTT expression in transgenic and wild-type mice thus any increase seen with the low stringency human probe is the result of an additional expression of h5-HTT m RNA. Transgenic mice show a 1.8-2.9 fold increase in mRNA in the raphé nuclei. The hippocampus shows no detectable expression in the wild type mice consistent with the results of Bengel *et al* (1997) and a low level in transgenic animals (*ca* $1/16^{th}$ of the maximum expression seen in the raphé). There is some evidence for the presence of 5-HTT mRNA in the hippocampus in adult rats (Lesch et al., 1993) and Hansson *et al* (1998) show that in adult rats the dentate gyrus shows a low number of 5-HTT positive cells. However, there are also a relatively high number of 5-HTT binding sites in the hippocampus of raphé origin (Sur et al., 1996). It is therefore interesting that these transgenic animals express transporter RNA in this area. It has been shown that 5-HTT RNA is expressed in the hippocampus CA1-CA3 and Dentate gyrus during development (Hansson et al., 1998), and it is possible that excess RNA at this time alters the configuration or long term expression in the hippocampus or that the normal level of expression in mice is so low as to be undetectable except by more sensitive techniques such as RT-PCR. The detected level was very low and it is possible that contamination from blood or other sources could have occurred during dissection. However, it is also possible that increased RNA in the raphé leads to axonal transport and like the 5-HTT protein this RNA is of raphé origin. It would be possible to test this by lesioning the raphé–hippocampal projection and re-analysing for RNA expression. However it is unlikely that even a 3-fold increase in RNA level would be sufficient to cause this effect.

Lesch *et al* (1993) also report RT-PCR detection of 5-HTT RNA expression in the cortex of rats. However *in situ* hybridisation studies have not detected expression in mice (Bengel et al., 1997) and this RT-PCR also consistently failed to detect 5-HTT RNA in the cortex of control animals. *In situ* studies also show that in wild type mice there was no expression in the cortex. In transgenic animals both the piriform cortex and olfactory nucleus show 5-HTT RNA expression. It is possible that

expression here occurs at very low levels or under certain physiological situations in these areas in wild-type animals and that this expression is an increase of a normally present phenomenon. It is also feasible that as both these areas have a serotonergic input (Hansson et al., 1998) the hypothesis mentioned above for the hippocampus applies and a disturbance in expression during development produces an alteration in adult RNA expression. Possibly the normal off-switch fails.

These results show that the human transgene is being expressed in these mice and confirms that the expression of the 5-HTT is targeted to certain regions. The presence of the extra DNA leads to increased RNA production suggesting that the expression of the 5-HTT is not regulated at this level (although it is possible that the extra RNA is not translated). It is also possible that the pattern of expression differs slightly in humans and mice, particularly in the cortex.

The expression of the human 5-HTT protein is studied in the next chapter.

Chapter 4

Pharmacological characterisation of 5-HTT protein expression

4.1 Introduction

The previous chapter described the expression of human 5-HTT RNA in transgenic mice, but this expression does not necessarily mean that the RNA is translated into 5-HTT protein. Three possible scenarios could affect protein expression:

1) the human protein is not expressed;
2) the human protein is expressed and the endogenous mouse protein is downregulated so that total 5-HTT protein levels remain constant; or
3) the human protein is expressed in addition to normal endogenous expression.

This last case is probably the most likely and would be the most useful model for serotonergic disturbance. However, the 5-HT system has many regulatory elements that may prevent over expression.

Two strategies were used to study 5-HTT protein expression. Firstly, the expression was quantified in membranes from the cortex and brainstem of transgenic and wild-type mice. The tritiated form of the SSRI citalopram ([^{3}H]citalopram), which has a high specificity for the 5-HTT, was used to measure 5-HTT protein expression in membranes. Citalopram recognises both human and mouse protein; therefore, in order to confirm human expression a pharmacological profile strategy was used. Since the human, mouse and rat 5-HTT have slightly differing affinities for antidepressants (Barker and Blakely, 1998; Barker et al., 1994; Tatsumi et al., 1997), the pharmacological profile and inhibition constants for different drugs in wild-type and transgenic mice can be compared, thus providing information about human 5-HTT protein expression and allowing the contribution of the human protein to be estimated. The affinities of antidepressants for the 5-HTT are described in the introduction (Section 1.2.1). Finally, the binding of [^{3}H]citalopram to the 5-HTT

was localised in brain sections from wild-type and transgenic mice to provide an expression pattern for the mouse and human protein, respectively.

Competition studies were used for the membrane binding and pharmacological profiles. Done at equilibrium conditions, these studies use a fixed concentration of radio-labeled ligand and sample tissue in the presence of a varying concentration of unlabelled drug, which binds to the same site as the ligand. Thus, as the concentration of competing drug increases, by the law of mass action, more of it binds to the protein and less radio-labeled ligand is bound (see Section 4.2.5).

In order to find the drugs with different affinities for human and mouse protein a comparison was first made between human, mouse and rat proteins. Assays were done on membrane preparations from mouse brains. As human brains were not available for comparison, human platelets were obtained (with thanks to the Rayne Laboratory, University of Edinburgh). To ensure that the comparison between platelet membranes and brain membranes was valid, platelets were also extracted from rats and the pharmacology compared to that in rat cortex. Because of technical constraints, it was not possible to obtain platelets from mice.

Saturation studies with increasing concentrations of unlabelled citalopram in the presence of a fixed quantity of [^{3}H]citalopram were then used to study the level of protein expression and the dissociation constant (K_D) of citalopram, which was found to differ between human and mouse proteins. Competition curves with other unlabelled antidepressants were also used to compare the pharmacology in transgenic and wild-type mice.

4.2 Methods

4.2.1 Membrane preparation from mouse and rat brain

Animals were killed by cervical dislocation followed by decapitation. Brains were removed, placed immediately in ice-cold 0.9% saline, then dissected as quickly as

possible. Regions taken were a) the cerebral cortex, b) brainstem and midbrain, and c) cerebellum. Tissues were rolled on filter paper to remove blood vessels and the tissue samples weighed. Rat brain samples were pooled but individual mouse brains were processed separately in case transgenic animals had variable expression levels. All solutions were kept on ice.

The tissues were homogenised (800 rpm) with a glass-teflon homogeniser (with 80μm clearance) in 10 (v/w) of 50mM Tris HCl (pH 7.4), diluted to 50 volumes with 50mM Tris HCl (pH 7.4) and centrifuged at 30000g for 10 minutes at 4°C to isolate the membrane fragments. The pellet was resuspended (50mM Tris HCl, pH 7.4), centrifuged (30000g, 10 minutes at 4°C), resuspended as before and incubated at 37°C for 15 minutes to remove endogenous bound 5-H T. The membranes were centrifuged as before and re-homogenised in 25 vols of 50mM Tris HCl, pH 7.4 and aliquoted before storage at –20°C. Membranes were stored for at least 1 week before use and for no longer than 6 weeks.

4.2.2 Preparation of rat platelet-rich plasma

Platelet-rich plasma (PRP) was obtained by adaptation of the method of Gordon and Olverman (Gordon and Olverman, 1978). Six female Spraque Dawley rats were anaesthetised with 4% halothane in oxygen and nitrous oxide (30:70 v:v). Blood, obtained by puncture of the *vena cava* into syringes primed with tri-sodium citrate, was mixed with one part 3.1% tri-sodium citrate per nine parts of blood to prevent coagulation. Blood samples were centrifuged at 2300 rpm (800g) for 2 minutes at room temperature (RT) and the top layer containing PRP removed carefully. This procedure was repeated twice more, and the PRP thus obtained was pooled. 7-9 ml of blood was obtained per rat, which generated approximately 3-5 ml of PRP.

4.2.3 Preparation of membranes from rat and human PRP

Rat PRP was obtained as above. Human PRP was obtained from the blood of volunteer control subjects as a by-product of lymphocyte extraction in the presence

of heparin as an anticoagulant, (Rayne Laboratory, Department of Biomedical Sciences, University of Edinburgh). Approximately 75 ml samples of PRP were obtained.

Rat PRP was then centrifuged in a microfuge at 15,000 rpm for 20 minutes. The pellets were re-suspended in Tris buffer (10mM Tris HCl, 5mM EDTA) at 200µl per 10 ml of original sample. Human PRP was centrifuged at 17,000g or 14,500 rpm for 20 minutes and the resulting pellet re-suspended in Tris buffer at 50 ml per 50 ml PRP. The resulting pellets were then homogenised on ice using an Ultra-Turrax homogeniser for 10 seconds, twice. Samples were incubated on ice for 1 hour to lyse the platelets, frozen for at least 30 minutes or overnight at –20°C to complete the lysis, thawed on ice and made up to 15 ml (rat) or 50 ml (human) with ice-cold 50mM Tris HCl.

Membranes were prepared as for brain membranes described above except that the incubation at 37°C to remove endogenous 5-HT was extended to two incubations of 30 minutes each. Membranes were isolated by centrifugation (10 minutes x 18000rpm for rat or 30000g for human samples. These conditions were used throughout.) and re-suspended by homogenisation in 5 ml (rat) or 50 ml (human) of 50mM Tris HCl. Rat samples were further diluted to 10 ml for ease of handling.

The centrifugation and re-suspension steps were repeated and followed by a cycle of incubation at 37°C for both human and rat platelet membranes (37°C, 30 minutes), centrifugation (10 minutes, 18000 rpm/30,000g), and re-suspension (10ml 50mM Tris HCl) to remove endogenous 5-HT. This incubation and centrifugation cycle was repeated. The incubation at 37°C was increased from the 10 minutes used for brain membranes described above as this was insufficient to release all the endogenous bound 5-HT from the platelets, which inhibited [^{3}H]citalopram binding.

After the second incubation at 37°C and centrifugation, the platelet membranes from individual rats were re-suspended in a final volume of 5 ml 50mM Tris HCl per 9 ml of original blood, and the human platelet membranes were re-suspended in 10 ml 50

mM Tris HCl per 50 ml PRP. Samples were frozen as 1 ml aliquots at –20°C until needed. On the day of assay the membranes were thawed on ice and diluted as necessary in assay buffer. Rat membranes were diluted to 50-60 volumes, mouse membranes to 40-50 volumes and human and rat platelets to 30 volumes.

4.2.4 Protein assays

The amount of protein was determined by the Bradford protein assay (Bradford, 1976). A serial dilution of bovine serum albumin (BSA) 10-200 µg/ml was used to generate a standard curve. 250 µl of Bradford reagent (0.1% Coomassie blue (w/v), 5% of 95% ethanol (v/v), 10% of 85% orthophosphoric acid, twice filtered through Whatman filter paper and stored at 4°C) was added to a 50 µl sample of the standards, or test sample, in a 96-well plate (Corning). The plates were incubated in the dark for 30 minutes before reading. A Dynatech plate reader was used to measure absorbance; samples were shaken for 20 seconds to mix and the absorbance was measured at an optical density of 595 nm (OD 595). OD 595 measurements for the BSA standards were fitted through the linear part of the curve to a straight line (Origin 41 for Windows). The amount of protein in each sample was then measured by interpolation.

4.2.5 [^{3}H]Citalopram binding assays

These studies had three objectives: firstly, to compare the pharmacological profiles of the human, rat and mouse transporters using cortical and platelet derived membranes; secondly, to compare [^{3}H]citalopram binding in wild-type and transgenic mice to determine 5-HTT protein expression levels; and thirdly, to compare the pharmacological profiles of wild-type and human 5-HTT (h5-HTT) transgenic mice to determine the contribution of human transporters.

Competition studies are carried out in the presence of a fixed concentration of radio-ligand and membrane under equilibrium conditions. The concentration of the competing drug is varied. Thus, as the concentration of competing drug increases

the amount of radio-labeled drug bound decreases, such that occupancy by labelled ligand A and competitive antagonist B can be defined by :

$$P_A = \frac{x_A / K_A}{x_A / K_A + x_B / K_B + 1} \qquad (1)$$

where P_A = occupancy by radio-labelled ligand; x_A = concentration of radio-ligand; x_B = concentration of inhibitor; K_A = equilibrium constant for the labelled ligand; and K_B equilibrium constant for the inhibitor.

Stock solutions (10 mM) of the drugs used, sufficient for all the experiments, were made in dH_2O and stored at –20°C until diluted in assay buffer on the day of the experiment. [^{3}H]citalopram (New England Nuclear) was diluted in dH_2O and stored under liquid nitrogen in aliquots sufficient for one experiment until needed. Assays consisted of duplicate tubes to determine total and non-specific binding and ten sets of duplicate tubes of competing drug at increasing concentrations. The binding assays were carried out in 5 ml round bottom polypropylene tubes (Sterilin (RT35), UK).

Membrane binding assays were carried out by pre-incubating 100 µl of buffer or test drug, 300 µl of assay buffer (50mM Tris HCl, 120mM NaCl, 5mM KCl, pH 7.4),and 100 µl of [^{3}H]citalopram (0.25 nM, 82-85 Ci/mmol) for 2 minutes at 25°C. Non-specific binding was determined in the presence of a concentration of the test drug at least 100-fold the affinity of the drug for the transporter. Binding was initiated by the addition of 500µl of the diluted membrane suspension to give a final volume of 1 ml. Incubation was continued for 1 hour to ensure that equilibrium was reached (equilibrium for citalopram was 30 minutes). Bound and free ligand were separated using a Brandel harvester over Whatman GF/B filters pre-wetted for 1 hour in assay buffer containing 0.05 % polyethyleneimine (PEI). Following two rapid 2 ml washes with assay buffer, the filter disks were transferred to scintillation vials and incubated for at least 10 minutes with 100 µl of 100% formic acid to aid dissolution. Emulsifier safe scintillant (2.5 ml) was added and equilibrated overnight. Radioactivity was determined in a Packard 2500TR scintillation counter with

automatic quench correction (counting efficiency approximately 50%). Samples were counted for 4 minutes and results expressed as disintegrations per minute (dpm) per sample. The concentration of the ligand for each binding assay was calculated from eight samples of the radioactivity added to the assay.

To compare the pharmacological profiles of the transgenic and wild-type mice, several competing drugs were used (see Table 4.2.1). In general a range of dilutions of half a log unit were used and the range of concentrations was selected to bracket the IC_{50} of the competing drug for the 5-HTT. Where possible, more closely space dilutions of the competing drug were included around the IC_{50} value to reduce the experimental error. When the competing drug is the unlabelled form of the radio-ligand used, in this experiment unlabelled citalopram, the graph of displaced binding versus concentration of competing ligand is a saturation curve. The data from these curves allow the calculation of the equilibrium dissociation constant ($K_{D)}$ and number of binding sites (B_{max}). Data was analysed with GraphPad Prism 3 (Mac) using non-linear regression analysis for curve fitting with the equation:

$$Y=\text{bottom} +(\text{top-bottom})/(1+10\text{^}((\log IC_{50}\text{-}X) \text{ x Hill Slope})) \quad (2)$$

where the top and bottom are the Y values at the top and bottom plateaus of the curve. Results were a logistic fit if the Hill slope equaled 1, which would be consistent with a one-site fit. To calculate the K_D, the dissociation constant at the 5-HTT and $B_{max,}$ the density of the available binding sites for citalopram, the following equations were used. IC_{50} (the concentration of ligand at which 50% of the maximal binding occurs) is an experimentally defined parameter, measured from the competition curve. As this constant depends upon the concentration of labelled ligand present, it can be converted to a measurement of the dissociation constant as follows:

$$K_D = IC_{50} - [^3H]\text{ligand concentration} \quad (3)$$

where the IC_{50} is that of the unlabelled ligand and the [^{3}H] ligand concentration in the assay determined from the standards.

The density of binding sites (B_{max} values in fmol/mg protein) were calculated by converting the specific binding (dpm) of the radio-ligand bound into the number of moles using the specific activity of the [^{3}H]citalopram and the following equation:

$$B_{max} = (b \times IC_{50}) / ([^3H]\text{-}[ligand] \times Pr) \qquad (4)$$

where b is the specific binding in fmoles at the [^{3}H] citalopram concentration used in the assay in the absence of inhibitor, IC_{50} is the concentration of the unlabelled ligand giving 50% inhibition, [^{3}H]-[ligand] is the concentration of radio-ligand in the assay and Pr is the amount of protein in the assay in mg.

As the IC_{50} is an experimentally derived parameter, where possible, an inhibition constant (K_i), which is dependent on radio-ligand concentration, was calculated according to the Cheng-Prussoff approximation for all inhibitors other than the cold form of the ligand (Cheng and Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + \left([^3H] - [ligand] / K_D \right)} \qquad (5)$$

A Hill coefficient far away from 1 indicates there is not one binding site, and is evidence for the presence of two sites. However, if it is not possible to separate into two sites, then the K_i value will appear to be altered and this would not be a valid measurement. In this case, the IC_{50} is determined.

Data analysis was by GraphPad prism 3(Mac) and Instat 2.03 using ANOVA followed by the appropriate test as recommended by prism- Dunnet's post analysis where one transgenic line was compared to control and Tukey's test where more than one variable was compared, as it is considered more appropriate for large data sets than Bonferronis.

DRUG	TRANSPORTER	CONCENTRATION IN ASSAY	PREDICTED IC50 (RAT)
CITALOPRAM	5-HT	0.001, 0.01, 0.1 ,0.2 ,0.5 ,1 ,2 ,5 ,10 ,30 ,100 (nM)	1nM
CLOMIPRAMINE	5-HT /NA	0.001, 0.01, 0.1 ,0.2 ,0.5 ,1 ,2 ,5 ,10 ,30 ,100 (nM)	2nM
FLUOXETINE	5-HT	0.01, 0.1, 0.2, 0.5, 1, 2, 5, 10, 30, 100, 1000 (nM)	7 nM
PAROXETINE	5-HT	0.0001, 0.001, 0.01 , 0.02, 0.05, 0.1, 0.2, 0.5, 1, 3, 10 (nM)	0.1nM
DESIPRAMINE	NA/5-HT	0.0002, 0.002, 0.02, 0.05, 0.1, 0.2, 0.5 ,1, 20, 5 ,20 (μM)	0.2μM
NOMIFENSINE	DA/NA	0.001 , 0.01, 0.1, 0.2, 0.5, 1, 2, 5, 10, 30, 100 (μM)	1μM

Table 4.2.1 Drugs used for competition binding studies and their pharmacological profiles. Predicted IC_{50} values are from preliminary experiments in rat cortical membranes and agree with those in the literature (Barker, Kimmel et al. 1994; Barker and Blakely 1998).

4.2.6 Localisation of 5-HTT protein expression in the brain of transgenic mice by in vitro autoradiography with [^{3}H]citalopram

In order to study the possible change in 5-HTT protein expression in the cortex of transgenic mice, [^{3}H]citalopram autoradiography was used in coronal sections of transgenic mouse brain. The method and results described are from a preliminary experiment with a range of [^{3}H]citalopram concentrations, a picture of the localisation pattern in the cortex was attained and thus some of the experimental results are included in this thesis.

4.2.7 In vitro autoradiographic [^{3}H]citalopram method and analysis

Six wild-type and six A102.3 mouse brains were processed, and coronal sections cut using a cryostat as for *in situ* hybridisation (See section 3.2.4). Sections were maintained at –70°C until used. Three adjacent sections were used for each concentration, two as duplicate determinations of total binding and one as non-specific binding

For autoradiographic studies, sections were allowed to equilibrate to RT. Slides were preincubated at RT for 15 minutes in assay buffer (see Table 4.2.2) and incubated in the presence of [^{3}H]citalopram for 1 hour (non-specific binding was measured in the presence of 10^{-6}M imipramine (which at this concentration blocks the 5-HTT as well as NET and DAT), on adjacent sections on a separate slide. The slides were then dipped in buffer at 4°C to remove excess ligand, and washed twice for 10 minutes in buffer at 4°C. Any salt deposit was then removed by dipping the slides in distilled water at 4°C before drying them in a cold air stream. Slides were then apposed, along with [^{3}H]-microscales calibrated to nCi/mg (Amersham) to [^{3}H]-sensitive Hyperfilm (Amersham), in X-ray cassettes for 8 weeks at 4°C. Films were developed according to the manufacturer's instructions and concentrations in fmol.mg^{-1} were calculated by the image analysis software using the specific activity of the [^{3}H]citalopram used in the experiment.

[^{3}H]ligand	Ci/mmol	[range] (nM)	Assay Buffer	°C	Time
Citalopram	82-85	0.02-20	50mM Tris HCl, 120mM NaCl, 5mM KCl(pH 7.4)	RT	1 hr

Table 4.2.2 Conditions for [^{3}H]citalopram autoradiography

Autoradiograms were analysed using a computer-based image analysis program (MCID/M4, Imaging Research Inc). After background subtraction, optical density (OD) values were converted to fmol.mg^{-1} tissue equivalent using standard curves of OD versus radioactivity in fmol.mg^{-1} from the tritium standards. OD measurements were made by selecting a suitable measuring tool, or box, of appropriate dimension, overlaying the structure of interest, and measuring the optical density of that area. Measurements were made bilaterally on two sections for each concentration. The same overlay was then used on the adjacent non-specific binding section. Specific binding was determined by subtraction of non-specific binding from the respective totals.

4.3 Results

4.3.1 Comparison of the pharmacological profiles of human, mouse, and rat 5-HTT.

The transgenic mice contain both human and mouse RNA. It was therefore necessary to establish the pharmacology of both the human and mouse transporters in isolation. As human brain tissue is difficult to obtain, platelet transporters with a high degree of pharmacological similarity to those in the brain were used. Since mouse platelets could not be obtained easily, membranes from rat cortex and platelets were compared to confirm that the 5-HTT in platelets and brain tissue is interchangeable. All experiments were conducted in parallel.

There was no significant difference between the binding affinities for 5-HTT for any of the drugs tested in rat brain and platelets (Table 4.3.1.1). Taken together with previously published studies in human tissue and expressed cell lines, this finding

	Rat cortex (6)		Rat platelets(2)		Mouse cortex (4)		Human platelets (2)	
	K_D/K_i (nM)	nH	K_D/K_i (nM)	nH	K_D/K_i (nM)	nH	K_D/K_i (nM)	nH
Citalopram	1.43±0.26	1	1.17±0.26	1.1	1.24±0.35	1.05	**3.53±0.26	0.9
Clomipramine	2.26±0.43	0.98	0.74	1	1.6±0.42	0.96	0.6	0.46
Fluoxetine	8.05±1.6	1.1	5.41±4.4	1	6.87±1.06	0.91	5.08±1.33	1.15
Paroxetine	0.21±0.08	1.2	0.09	1.27	0.1	0.64	0.11	0.73
DMI	*313 ±24	0.9	201.38	1.2	237±21	1.36	***65.8±21	1.25
Nomifensine	1161.2±60	0.7	890.15	0.9	1624.6	0.85	6025.3	0.9

Table 4.3.1.1 Pharmacology of [^{3}H] citalopram binding in mouse, rat and human.

The affinity values were determined from the inhibition of 0.25mM [^{3}H] citalopram binding in membranes from rat cortex, rat platelets, human platelets and mouse cortex. Data is expressed as mean ± SEM. Statistical analysis ANOVA followed by Student-Newman-Keuls or Tukeys multiple pair-wise analysis *=$p<0.05$ **=$p<0.01$ ***=$p<0.001$ compared to mouse where figures were available using Instat2.03 . (2)= n. Where no SEM is given n=1.

confirms that platelet transporters are sufficiently similar to those in the CNS for use as a model of human transporter function.

Whilst there were no significant differences in the affinity of citalopram between the rat and mouse 5-HTT or between the rat cortex and platelets, there was a slight but statistically significant change in the affinity for citalopram between human and rat or mouse 5-HTT. The human transporter has a higher K_D (3.53 nM) compared to the mouse (1.34 nM), a difference that was significant ($p<0.01$). The increase in K_D was small, but the slight reduction in affinity for citalopram is important, as a shift toward the human K_D in transgenics will indicate expression of the human variant. However, such an increase will also affect the possible analysis of the competition studies as the inhibition constant K_i is derived from the K_D value of the labelled ligand. In transgenic mice where the K_D is altered, the K_i cannot be determined accurately.

A range of amine uptake inhibitors was tested in all species (Table 4.3.1.1). The SSRI paroxetine had the highest affinity, with a K_i value of 0.1-0.2 nM. The other SSRIs, citalopram and fluoxetine, were also potent but the dibenzazepine clomipramine, which has affinity for both the 5-HTT and noradrenaline (NA) transporter, was more potent than fluoxetine. Desmethylimipramine (DMI), which has affinity for both NA and 5-HT transporters, was less potent than the SSRIs as was the selective NA uptake inhibitor, nomifensine. For all the tissues tested, the order of potency for inhibition of [^{3}H]citalopram binding (K_i) was

Paroxetine>Citalopram>Clomipramine>Fluoxetine>DMI>Nomifensine.

The inhibition constants for fluoxetine, DMI and nomifensine had possibly differed between human and mouse transporters in the preliminary study (Figure 4.3.1.1). Two of these drugs were tested further (n=4-6) – fluoxetine and DMI. Further studies indicated that only DMI had a significantly different inhibition profile in the mouse and human (K_i = 237±21 and 66±21 nM, respectively; $p< 0.001$.) (Table 4.3.1.1, Figure 4.3.1.2). These values agree with the published inhibition constants

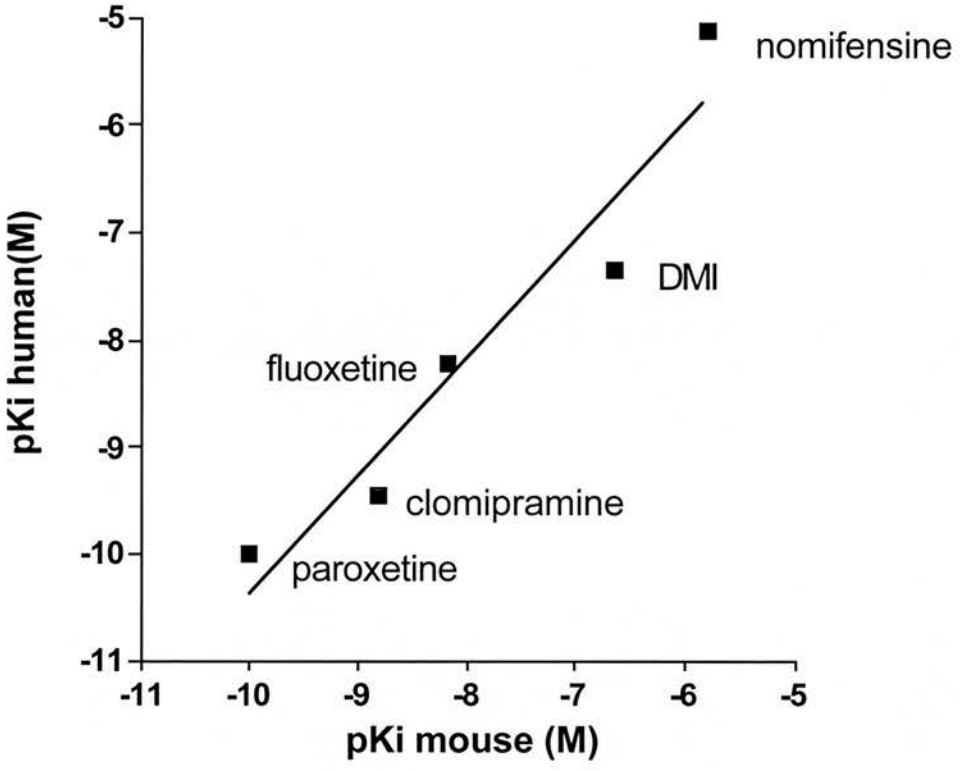

Figure 4.3.1.1 Correlation of affinities of [^{3}H]Citalopram binding in human platelet and mouse cortical membranes. Affinities for Inhibition were determined from at least two experiments (table 4.3.2.1). The correlation coefficient 9r0 obtained by linear regression and correlation analyses of the data was 0.9227 $p<0.01$ with a slope of 1.1±0.18 which was not significantly different from one.

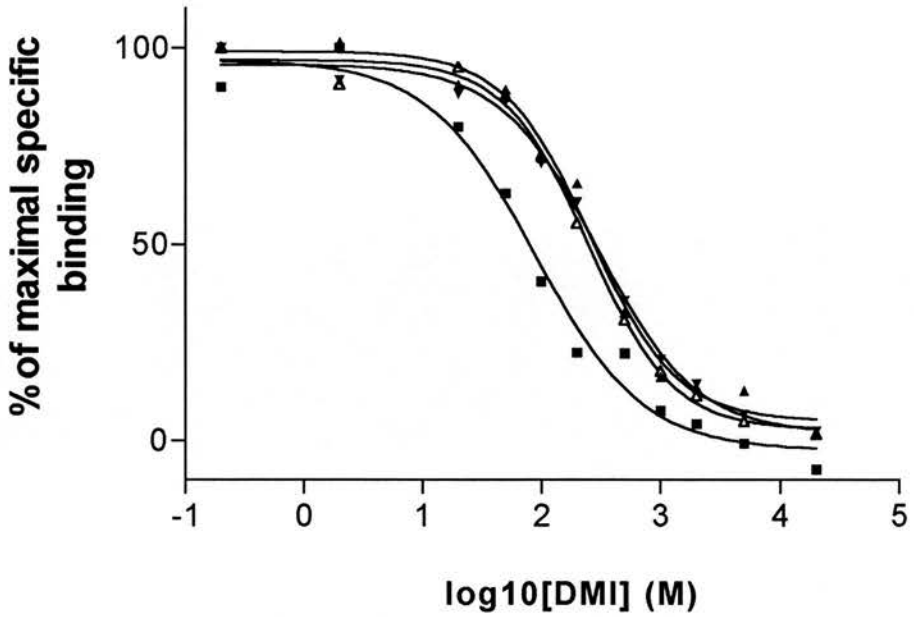

Figure 4.3.1.2 inhibition of [3H]citalopram binding by DMI in membranes from human, mouse and rat tissues.

▾ = rat cortex ▵ = rat plt ▪ =human ▴ = mouse

(Table 4.3.1.1, Figure 4.3.1.2) for inhibition of [^{3}H]5-HT binding by DMI in rat and human transporters in heterologous expression systems (Barker and Blakely, 1998), where the tricyclic antidepressants imipramine and DMI were found to be more potent at the human versus the rat transporter.

All the drugs used bound to a single site. The Hill coefficient did not deviate significantly from unity. This finding is important as it might be expected that the Hill coefficient would be less than one in transgenic animals if the K_i were significantly different in the two species. Those drugs with different affinities for the human and mouse transporter, such as DMI, are useful for identifying the presence of both human and mouse protein in the transgenics as a shift in the affinity towards that of the human suggests that any extra protein present is the human form.

4.3.2 Affinity for citalopram and binding site density in the cortex and brainstem membranes of wild-type and transgenic mice.

In wild-type animals the K_D for citalopram was 1 nM and the Hill coefficient did not differ from unity, showing the presence of only one binding site. The B_{max} in the cortex was 868 fmol.mg^{-1}protein. There was a small (non-significant) increase in the disassociation constant (K_D) for citalopram in the cortex of both lines of transgenic mice tested compared to wild-type animals. This finding suggests a shift towards the human value and thus the presence of the human transporter. In addition to the change in K_D, the Hill coefficient (nH), which is a measure of the slope of the curve, was less than unity in the transgenic mice. This observation is consistent with the presence of two binding sites with different affinities.

There was a 2-3 fold increase in the B_{max} (available binding sites) in both transgenic lines tested compared to wild-type mice, confirming that the 5-HTT is over-expressed in these transgenic mice. This increase is greater in line A102.3 than A102.2, suggesting that the two lines express different amounts of the human protein. A102.2 expresses 50% human, 50% mouse, whilst line A102.3 expresses 33% mouse and 66% human protein.

	B_{max} fmol.mg^{-1} protein	K_D (nM)	nH
F1 (4)	868±309	1.19±0.44	1.05±0.16
A102.2(6)	1829±388	1.28±0.29	0.74±0.09
A102.3(4)	*2790±571	1.60±0.26	0.58±0.05

Table 4.3.2.1 Affinity and binding site density for [^{3}H]citalopram in the cortex of wild-type and transgenic mice. * $p<0.05$, ** $p<0.01$ by ANOVA followed by Dunnet's pair-wise comparison. Data are expressed as the mean ± standard error (n= 4).

Figure 4.3.2.1 shows a model that explains the effect of two binding sites with different affinities on the binding curve. When two sites are present, the curve generated is the product of two curves, one for each site, added together. If the difference in affinities for the two sites is less than an order of magnitude, as in this case, the two curves cannot be separated by a two-site fit. However using a logistic fit and looking at the Hill coefficient as an indirect measure; the slope of the curve is altered, becoming flattened, and the slope is reduced from one.

There were no significant differences between the K_D and B_{max} of brainstem tissue from wild-type and transgenic mice. Although these values increased, their differences did not reach significance.

	B_{max} fmol.mg^{-1} protein	K_D (nM)	nH
F1	2821±627	1.29±0.21	1.03±0.08
A102.2	3108±290	1.34±0.28	0.85±0.15
A102.3	3848±552	1.75±0.14	0.89±0.11

Table 4.3.2.2 Affinity and binding site density for [^{3}H]citalopram in the brainstem of wild-type and transgenic mice. * $p<0.05$, ** $p<0.01$ by ANOVA followed by Dunnet's pair-wise comparison (Instat 2.03). Data are expressed as the mean ± standard error of at least 4 animals.

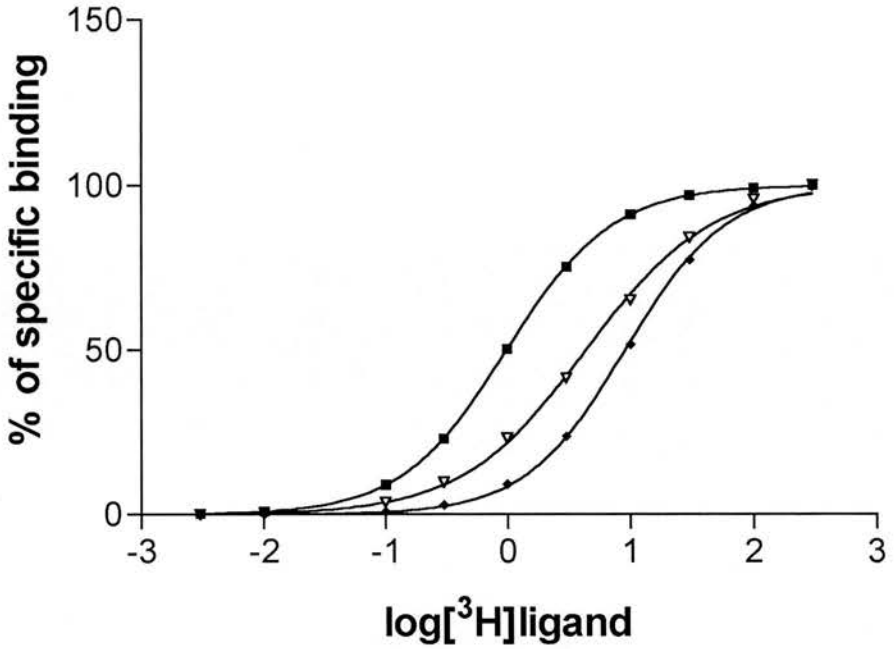

Figure 4.3.2.1 Model showing the effect of the presence of two binding sites with different affinities for the ligand on the shape of a saturation curve. Site A has and KD = 1 and site B a kD = 10. Both have hill coefficients of 1. When the two sites are expressed in the same system at equal levels (A+B) it can be seen that the curve becomes flattened. In this example the measured kD of A+B is 4.39 and the Hill coefficient 0.856. ▪ = A • = B ∇= A+B

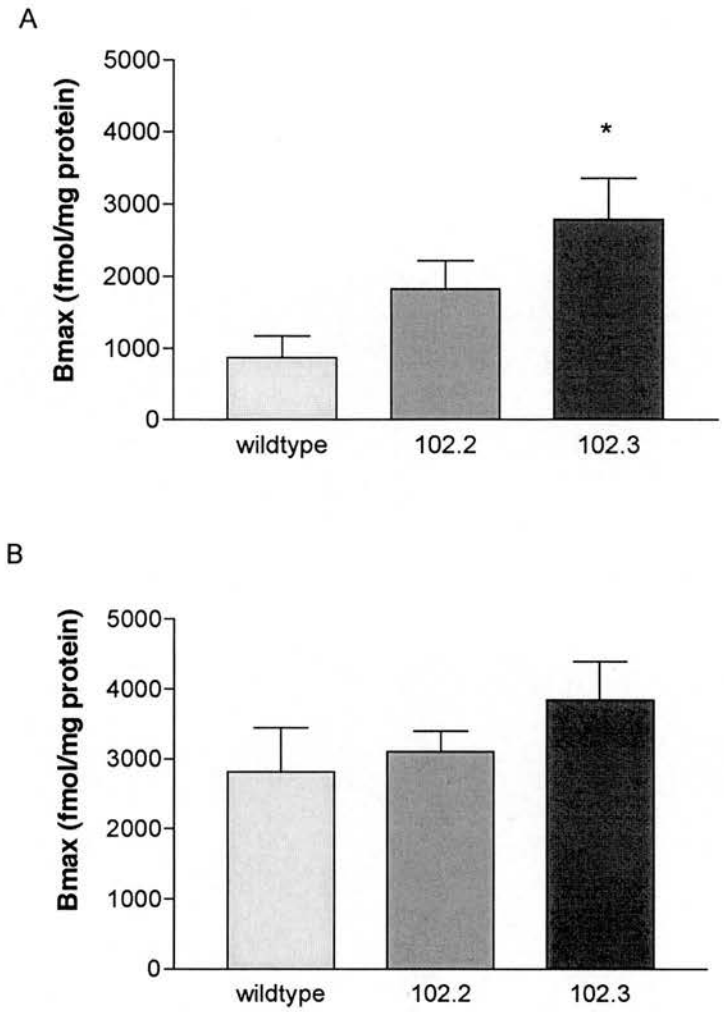

Figure 4.3.2.2 Binding site density in A) the cortex and B) the brainstem of transgenic and wildtype mice.

The expression of 5-HTT in the brainstem of wild-type mice is significantly higher than in the cortex, which could possibly mask any changes in K_D or B_{max} (Figure 4.3.2.2.). The cortex has 5-HTT protein from DRN neurons in the brainstem as well as the additional expression indicated by the presence of RNA in the piriform cortex. Thus two factors may be involved in the 5-HTT increase seen in the cortex. Further experiments will concentrate on the cortex where the expression is easiest to isolate. There was no detectable signal in any of the cerebellar membranes of wild-type or transgenic mice.

4.3.3 Pharmacology of antidepressant binding in the cortex and brainstem of wild-type and transgenic mice

The presence of two sites with differing affinities for the [^{3}H]citalopram results in a slight change in the K_D. This change in itself is not significant, but as the inhibition constant for the competing drug is related to the K_D it may cause apparent shift in the K_i,

$$K_i = \frac{IC_{50}}{1 + \left([^3H] - [ligand] / K_D \right)} \qquad (6)$$

it is therefore possible only to compare the IC_{50} values for the competing drugs and not the K_i values. Tables 4.3.3.1 and 4.3.3.2 show the pharmacological profile for cortex and brainstem membranes, respectively.

In both wild-type mice and in transgenic lines A102.2 and A102.3, the order of potency remains the same with clomipramine > fluoxetine > DMI. In cortical membranes the affinity of DMI was higher in transgenic mice than in wild-type controls ($p<0.05$). Like the change in affinity for citalopram, this increase was slightly higher in line A102.3 than A102.2. There was no significant change in the IC_{50} for fluoxetine or clomipramine between wild-type and transgenic mice.

	F1		102.2		102.3		human	
	IC_{50}	nH	IC_{50}	nH	IC_{50}	nH	IC_{50}	nH
Citalopram	1.50±0.44	1.05	1.60 ±0.3	0.74	1.91±0.26	0.58	**3.71±0.2	0.9
DMI	#308.2±40	0.92	**148.3±16	1.04	***112.3±7.5	1.01	***67.4±19.5	1.25
Clomipramine	2.58±0.7	1.11	1.29±0.3	1.07	0.87±0.09	1.26	0.36	0.46
Fluoxetine	6.78±1.06	0.84	*.43±0.5	1.38	3.65±0.49	1.16	5.38±1.5	1.15

Table 4.3.3.1 Pharmacological profile of amine uptake inhibitors in the cortex of wildtype and transgenic mice. *= $p<0.05$ **=$p<0.01$ ***= $p<0.001$ compared to F1 mice #=$p<0.001$ compared to human by ANOVA followed by Tukey's pair-wise comparison (n=4-6). Data expressed as mean ± SEM

	F1		102.2		102.3		human	
	IC_{50}	nH	IC_{50}	nH	IC_{50}	nH	IC_{50}	nH
Citalopram	1.55±0.21	1.03	1.6±0.28	0.85	2.01±0.14	0.89	**3.71±0.2	0.9
DMI	#180±9.99	1.11	#225.32 ±36	0.96	#217±32	1.22	*67.4±19.5	1.25
Clomipramine	1.71±0.28	1.11	1.70±0.4	1.31	1.23±0.15	1.37	0.36	0.46
Fluoxetine	5.14±.95	0.71	5.58±0.5	1.34	2.52±1.11	0.97	5.38±1.5	1.15

Table 4.3.3.2 Pharmacological profile of amine uptake inhibitors in the brainstem of wildtype and transgenic mice *= $p<0.05$ **=$p<0.01$ compared to F1 mice #= $p<0.05$ compared to human by ANOVA followed by Tukey's pair-wise comparison (n=4-6). Data expressed as mean ± SEM

In the brainstem there was no significant difference between wild-type and transgenic mice in the IC_{50} any of the drugs tested. However, the B_{max} and K_D for citalopram were increased in transgenic animals and the Hill slope was not far off unity, yet not quite one, suggesting that two sites are present. Nonetheless, the proportion of human 5-HTT expression may be lower in the brainstem than in the cortex. These changes were similar to those in the cortex and towards the human values. However, unlike the cortex there was no increase in the affinity of DMI in the brainstem of transgenic mice

4.3.4 In vitro localisation of cortical [^{3}H]citalopram binding.

The saturation curve for [^{3}H]citalopram was not very successful. There are several probable reasons. Firstly, the mouse brain is very small and so there was a considerable amount of variation in binding density as the first and last sections were not within the same brain areas. Secondly, since the same solution was used for all the sections and was thus re-used, at low ligand concentrations there was probably some ligand depletion by the end of the experiment. Three batches of slides were treated in each dish. Thus though there was a trend to increased binding in transgenic animals, the errors masked any likely significant result.

However, the main aim to localise [^{3}H]citalopram binding was achieved in both transgenic and wild-type mice. Figure 4.3.4.1 shows sections from the cortex of transgenic and wild-type mice. The pattern of binding was similar in both genotypes and was consistent with the published pattern of expression of 5-HT neurons. None of the cerebral cortical areas without 5-HTT expression in wild-type mice showed expression in transgenic mice. The piriform cortex did not show 5-HTT protein expression (Figure 4.3.4.1b) and the granular cell layer of the olfactory nucleus likewise did not show expression, despite the detection of RNA in these areas by *in situ* hybridisation.

Transgenic

Wild-type

Figure 4.3.4.1 Localisation of [^{3}H] citalopram binding in the cortex of wild type and transgenic mice

4.4 Discussion

4.4.1 Comparison of human platelet and mouse 5-HTT

As a result of the recent generation of knockout mice without a functional 5-HTT, there is a large body of published information on the effects of reduced 5-HTT activity and its interactions with 5-HT autoreceptors in mice. The 5-HTT knockout mouse is often cited as an animal model for human antidepressant use. There is also a long-used assumption that the platelet transporter is functionally interchangeable with the cerebral form, though considerable evidence suggests that regulation of the platelet 5-HTT does not necessarily reflect that in the brain. Despite this evidence, very few studies have directly compared mouse and human 5-HTT pharmacological profiles.

The results of this thesis research confirm that platelet and cortical membranes have identical pharmacology for all of the amine uptake inhibitors tested, in agreement with the literature. The comparison of all three species in parallel experiments had not been made prior to this study although rat and human 5-HTT have been compared in several studies. There is no difference between the pharmacological profile of the rat and mouse 5-HTT. There was, however, a difference between the affinities of some of the tricyclic antidepressants (TCAs) used for human and rodent transporters. In particular, the dibenzazepine DMI and possibly clomipramine had greater affinity for the human transporter, though this was not tested in detail. This finding agrees with previous studies in native platelet preparations (Wielosz et al., 1976) where clomipramine and imipramine were found to be more potent in human than rat platelets. More recent studies also found that the tricyclic antidepressants were more potent at binding to the human than the cloned rat transporter in a heterologous system where human and rat 5-HTT were transiently transfected into HeLa cells (Barker and Blakely, 1998; Blakely et al., 1991; Ramamoorthy et al., 1993a).

4.4.2 Comparison of transgenic and wild-type mice

Cortical membranes from transgenic mice exhibited a shift in the K_D of citalopram towards the human affinity rather than the mouse. There was a shift in the Hill coefficient away from unity, consistent with the presence of two sites with different affinities for the ligand. In addition there was an increased density of 5-HTT binding sites, which confirms an increased expression of 5-HTT in transgenic mice. The density of 5-HTT binding sites was greater in transgenic line A102.3 than A102.2, suggesting that the two lines express the protein at different levels in the cortex (B_{max} 2:1, 1.6:1, respectively). Consistent with this over-expression being due to the human protein, and in addition to the change in the Hill coefficient suggesting two sites, the pharmacological profile showed a shift in the potency K_i of DMI and clomipramine in the cortex towards the human values and significantly different from that in wild-type animals. As the tritiated ligand is specific for the 5-HTT, the change in DMI potency is not due to a change in NA transporter activity.

These differences were less pronounced in line A102.2 than A102.3. This observation agrees with the mRNA data, which showed that line A102.2 expressed 5-HTT mRNA at lower levels than A102.3. Although there are several possible reasons for the variability of expression, the most likely explanation is that the incorporation of different copy number of the YAC occurred in the different transgenic lines. It is also possible that the loss of part of the YAC in the transgene incorporated in line A102.2 is sufficient to influence expression. Additionally, the site of integration of the transgene into the mouse genome influences transgene expression.

In the brainstem, there was a trend towards increased expression, but less than that seen in the cortex. The less pronounced changes in the brainstem may be because the cortex has several sources of 5-HTT protein as it receives input from the DRN in the brainstem, as well as the hypothalamus and other forebrain regions. In studies of [^{3}H]citalopram binding in 5-HT lesioned animals (Lawrence *et al.*, 1993), hippocampal binding was abolished but cortical binding was only reduced by 50%,

suggesting that there is more than one source of 5-HTT protein in the cortex. In addition, *in situ* hybridisation studies (see Chapter 3) indicated that there was ectopic expression of the 5-HTT transgene in the cortex, but not in the brainstem. In the [^{3}H]citalopram autoradiographic experiments, protein expression was not seen in the same areas as human RNA localisation, suggesting that either the RNA is not functionally transcribed or that it is located only in the somatic regions of cells which may project elsewhere in the cortex. In dissecting the brain for tissue samples, care was taken to remove the hippocampus and striatum from the cerebral cortex sample.

The density of [^{3}H]citalopram binding sites was also lower in the cortex of wild-type mice than the brainstem sample. This finding agrees with the published reports on the distribution of the 5-HTT in rats (Lesch *et al.*, 1993). It is possible that the different brain areas have different capacities for increasing 5-HTT expression. Such putative differences may be important in studying the effects of increased 5-HTT on the behaviour and neurochemical effects of 5-HTT over-expression. An additional factor may be that the human 5-HTT is targeted differently and may be found in the cortex.

Though these studies show that 5-HTT protein is over-expressed in transgenic mice at nearly twice the endogenous level and that the protein is successfully targeted to the membrane, the findings do not guarantee that the protein is fully functional. On the other hand, the successful citalopram binding does suggest that at least part of the protein has normal conformation. These studies also do not provide any information about protein turnover or levels of cytosolic or malformed protein.

4.4.3 Summary

5-HTT transgenic mice over-express the 5-HTT protein (2.1-3.2 fold in the cortex and 1.1-1.3 fold in the brainstem). The shifts in K_D value and Hill coefficient away from unity and altered pharmacological profile show that this over-expression is due to the human form of the protein.

4.4.4 Further work

The localisation of the 5-HTT protein using saturating concentrations [^{3}H]citalopram autoradiography is continuing. In addition to this effort, 5-HT uptake studies to show that the over-expressed protein is definitely functional and immunocytochemistry with antibodies specific to human and mouse 5-HTT would help complete the picture of the human 5-HTT expression.

Chapter 5

Characterisation of the serotonergic neurochemistry of h5-HTT transgenic mice

This chapter reports the measurement of the concentrations of 5-HT, dopamine and their metabolites 5-HIAA and DOPAC in brain tissue samples from transgenic and wild-type mice. The aim was to see whether increasing the expression of the 5-HTT had any consequences for the animals' neurochemistry. Any changes seen in the 5-HT system would help to clarify the role of the transporter in regulation of serotonergic function and explain any behavioural changes observed in the over-expressing mice and in models of human depressive disorders. Such changes may also help to explain why increased 5-HTT levels have been linked to depression (Dahlstrom et al., 2000).

5.1 Introduction

The major proportion of 5-HT concentrations in tissue samples is in intracellular stores and is therefore dependent upon the interactions among 5-HT synthesis, re-uptake and storage, and metabolism. The following summarises the comprehensive description of 5-HT metabolism given in Chapter 1.

5-HT is synthesised within the neuron from the amino acid tryptophan, with the rate-limiting step being is the conversion of tryptophan to 5-Hydroxytryptophan by the enzyme tryptophan hydroxylase (Grahame-Smith, 1964). Tryptophan hydroxylase is not saturated at physiological conditions so the synthesis of 5-HT can be altered by the availability of tryptophan as well as alterations in enzyme activity (Hamon et al., 1981; Hamon and Glowinski, 1974). Tryptophan levels are mainly dependent upon dietary intake and are not easily regulated. The activity of tryptophan hydroxylase is regulated by both Ca^{2+}/calmodulin-dependent (Hamon et al., 1981; Kuhn and Lovenberg, 1982) and cAMP-dependent (Foguet et al., 1993) protein kinases that convert the non-active form tryptophan hydroxylase to the active enzyme.

This activation can be altered by 5-HT autoreceptors. 5-HT_{1A} receptors are negatively linked to adenyl cyclase but also to G_i-protein-regulated K^+. or Ca^{2+}-dependent ion channels and so may act through either protein kinase system (Starke et al., 1989; Williams et al., 1988). The terminal autoreceptor 5-HT_{1B} also inhibits 5-HT synthesis. Stimulation of the 5-HT_{1B} receptor markedly inhibits synthesis, and the blockade of 5-HT_{1B} receptors increases synthesis, indicating that endogenous 5-HT exerts a tonic effect on synthesis via this receptor (Moret and Briley, 1997a; Moret and Briley, 1997b; Stenfors et al., 2000; Stenfors et al., 2001). Both 5-HT_{1A} and 5-HT_{1B} receptors can regulate synthesis independently under experimental conditions (Barton and Hutson, 1999) but they are probably not the only receptors involved physiologically. SSRIs and tricyclic antidepressants increase extracellular 5-HT and inhibit pre-synaptic neuronal firing and 5-HT synthesis (Aghajanian, 1972; Carlsson and Lindqvist, 1978; Corrodi and Fuxe, 1969; Fuller et al., 1974; Gartside et al., 1995; Hjorth, 1993; Hjorth and Sharp, 1993; Hjorth et al., 1995; Invernizzi et al., 1992; Svensson, 1978). This acute effect depends on the presence of 5-HT and is not prevented by blockade of 5-HT_{1A}, 5-HT_{1B} or a combination of both receptors (Moret and Briley, 1997b; Stenfors et al., 2001). This suggests that there is another mechanism by which 5-HT release inhibits synthesis.

5-HT is stored in vesicles before release and on re-uptake 5-HT is either transported by the VMAT2 from the cytoplasm into vesicles and re-stored or broken down by the monoamine oxidase A/aldehyde dehydrogenase pathway (Cases et al., 1995; Celada et al., 1992). The most stable and easily measured product of this process is 5-Hydroxyindole-3-acetic acid (5-HIAA). 5-HT metabolism is controlled by the activity of the monoamine oxidase A (MAO-A) pathway. Increasing 5-HT concentrations in the cytoplasm will increase the amount of 5-HT metabolised by the enzyme. 5-HT itself may regulate MAO activity (Tyce, 1990). There is also some evidence that a polymorphism in MAO-A may differentially effect serotonin metabolism (Tyce, 1990). Mice in which monoamine oxidase A has been knocked out have up to nine times greater than normal tissue 5-HT concentrations (Cases et al., 1995), and MAO inhibitors increase 5-HT levels whilst reducing 5-HIAA levels

in rodents and humans. 5-HIAA can be measured in tissue samples with relative ease. The absolute concentration of 5-HIAA but more usefully the ratio of 5-HIAA/5-HT is a used as a measure of 5-HT turnover and activity.

The 5-HT and dopamine systems interact at several levels; it was therefore possible that altered 5-HT neurochemistry in turn affects dopamine concentrations. The most stable and easily measured metabolite of dopamine is 3,4-dihydroxyphenyl acetic acid (DOPAC), which was therefore included in the assays. If there were any apparent changes in DOPAC concentrations, then dopamine and DOPAC tissue concentrations were assayed together in a different assay.

Measuring the concentrations of both transmitter and its metabolites provides considerable information about the state of the transmitter system, particularly the feedback state. In the 5-HT system a decrease in absolute transmitter levels indicates either 1) a reduced reuptake efficiency, 2) a reduction in synthesis or 3) greater metabolism. A concurrent increase in 5-HIAA would suggest that greater metabolism was a factor. An increase in the ratio of 5-HIAA/5-HT would indicate greater 5-HT turnover and therefore greater release. It is thus of interest to compare the effect of 5-HTT over-expression on transmitter and metabolite concentrations to provide information about the status of, and the feed back in, the 5-HT system.

5.1.1 5-HT and 5-HTT concentrations in knockout mice

On neuronal stimulation in 5-HTT knockout mice, the release of 5-HT has been shown to be severely reduced and re-uptake abolished, in addition tissue 5-HT is depleted by 60-80%. A small, though statistically significant, increase in 5-HIAA (Bengel, 1998) was also seen, which further indicates the importance of 5-HTT in 5-HT homeostasis. 5-HT is usually metabolised by monoamine oxidase within the cell, but monoamine oxidase within the synaptic cleft has some affinity for 5-HT and may be the cause of the increased 5-HIAA. In 5-HTT knockout animals dopamine and noradrenaline (NA) and their metabolites were unaffected by the change in 5-HT, possibly due to adaptations during development. These 5-HTT knockout

animals survive but show other alterations in the 5-HT system: decreased 5-HT_{1A} receptor RNA and protein levels in the raphé nuclei and regional-specific reduced 5-HT_{1B} expression (Fabre et al., 2000); and regional-specific adaptive reduction of 5-HT_{2A} receptor expression (Rioux et al., 1999). The 5-HTT knockout mice also show abolition of the pharmacological induced decrease in 5-HT turnover caused by the 5-HT_{1A} agonist isipirone, and loss of the 5-HT release caused by 5-$HT_{1B/1D}$ antagonists (Fabre et al., 2000). Attenuation of the locomotor responses to ecstasy (Bengel, 1998) in these knockout mice confirms the marked alterations of both autoreceptor and postsynaptic receptor function and suggests a possible effect on the DA system in knockout mice. The knockout animals have been suggested as a model for chronic antidepressant treatment as the 5-HTT is blocked; however, 5-HT is an important developmental signal and so the lifetime loss of 5-HTT activity will lead to adaptive changes that are different from adult onset of 5-HTT blockade with antidepressants. Therefore, whilst the changes in receptor function in knockout mice are interesting as a study of 5-HT system, the comparisons with depressed patients have to be drawn with circumspection. 5-HT_{2A} receptor expression is a case in point: there are important periods during development where the ontogeny of 5-HT_2 receptors can be affected (Morilak and Ciaranello, 1993). In SSRI-treated rodents 5-HT_{2A} receptors are increased whilst in 5-HTT knockout animals 5-HT_{2A} receptor expression is decreased.

5-HT metabolism is also altered in depression where an increase in 5-HIAA (Martensson et al., 1989) and a decrease in 5-HT levels are seen in the CSF of patients. The most effective drugs for treating depression, TCAs and SSRIs, increase the level of 5-HT in rodents. This observation, along with the evidence of altered 5-HT metabolism in depressed patients, formed the basis of the working hypothesis that the biochemical basis of depression is a deficiency in the levels of available brain serotonin, particularly in the hippocampus, an area greatly involved in the regulation of mood and which receives high levels of serotonergic input from the DRN and MRN.

Recent evidence that 5-HTT levels are increased in some types of depressive patients (Dahlstrom et al., 2000) implies that transgenic animals over-expressing the 5-HTT may be a better model than knockout animals for changes in the human 5-HT system that lead up to clinical depression. In this research the concentration of 5-HT, its metabolite 5-HIAA and the dopamine metabolite DOPAC were measured in transgenic animals using high pressure liquid chromatography (HPLC) with electrochemical detection.

5.2 Methods

5.2.1 Dissection of tissue samples

Mice were killed by cervical dislocation and their brains quickly removed. Tissue samples from the cerebellum, brainstem, midbrain, basal ganglia, hypothalamus, hippocampus and frontal cortex were dissected quickly (about 10 minutes per mouse) and frozen on dry ice. Samples were stored at $-70^{\circ}C$ until required. Eight male h5-HTT transgenic mice, from line A102.3, with eight age-matched controls were used per group. The tissue areas were chosen to cover 1) the originating nuclei of 5-HT pathways (brainstem, midbrain), 2) forebrain regions with 5-HT inputs (hippocampus, frontal cortex, hypothalamus), 3) regions with low 5-HTT expression as a control (cerebellum), and 4) a region where the 5-HT system might alter dopamine (basal ganglia).

An initial coronal cut was made immediately anterior to the cerebellum and followed by a second cut to separate the brainstem and cerebellum. Care was taken to prevent contamination of the cerebellum sample with brainstem or spinal cord. The midbrain was then dissected away from the forebrain and the hippocampus identified and removed. Next a block of hypothalamic tissue, which extended from the anterior edge of the optic chiasm to the caudal edge of the mamillary body and laterally to the hypothalamic sulci, was removed. Finally an area corresponding to the basal ganglia and basal forebrain was removed according to stereotaxic co-ordinates in the mouse atlas of the brain (Franklin, 1997). This area includes the accumbens nucleus, ventral

pallidum and median forebrain bundle, islands of Calleja, and magnocellular preoptic nucleus.

5.2.2 HPLC detection of 5-HT, 5-HIAA and DOPAC

A maximum of four samples were processed at a time to avoid oxidative degradation of the amines. All samples from one brain region were analysed on the same day as HPLC machines are sensitive to daily fluctuations in temperature and pressure. 5-HT and 5-HIAA were assayed together to ensure that comparisons could be drawn between them. The assay conditions also produced a visible peak corresponding to DOPAC, which was also measured. Dopamine could not be measured in this assay.

Samples were analysed according to a variation of a previously reported method for the detection of monoamine metabolites (Zaczek and Coyle, 1982). Briefly, samples were weighed whilst frozen, then thawed in 500 µl cold 0.1M perchloric acid. Tissues were homogenised by ultrasonication for 30 seconds or by using a Ultra-Turrax homogeniser (for three 10-second bursts). The cerebellum was more difficult to homogenise and the process was therefore repeated for this region. The homogenate was centrifuged for 5 minutes to remove any solid matter and the top 200 µl of supernatant was removed and stored on ice to prevent solid matter re-suspending and clogging the column; the supernatant was protected from light to prevent degradation. An aliquot (50 µl) of this supernatant was then analysed for 5-HT, 5-HIAA and DOPAC by HPLC with electrochemical detection. 5-HT, 5-HIAA and DOPAC were resolved in the same run using a Microscorb column (C18 ODS 2, Rainin) and detection was by a BAS (LC-4) potentiometer with a working electrode set at +0.7 Volts. De-gassed mobile phase (methanol 12.5%, EDTA 0.03%, NaH_2PO_4 127mM, octane sulphonic acid 15mM, pH 4.0) was pumped at 1.2 ml/min using an HPLC pump.

Calibration was by an external standard (5 pmol each 5-HT, 5-HIAA, DOPAC in 0.1 M perchloric acid) and fresh standards were made each day from concentrated stock. Stock solutions of 10 mM for each of 5-HT, 5-HIAA, and DOPAC were dissolved in

dH_2O and stored at 4°C. Stock solutions were made fresh each week and were diluted on the day by serial dilution in 0.1M perchloric acid.

5.2.3 HPLC detection of dopamine, DOPAC and 5-HIAA

Those basal ganglia samples that showed a change in DOPAC concentrations in the 5-HT analysis described above were re-analysed for dopamine content in a separate assay. Supernatant from the tissue preparation was stored at 4°C overnight, then diluted 1/5 in 0.1 M cold perchloric acid. A 50µl aliquot of diluted supernatant was then assayed for DA, DOPAC and 5-HIAA using similar apparatus to that described for the 5-HT assay, but different mobile phase. Mobile phase composition was methanol 16.7%, EDTA 0.03%, NaH2Po4 116mM, octane sulphonic acid 24mM, pH 3.85. De-gassed mobile phase was pumped at 1.2 ml/min using an HPLC pump.

Calibration was by an external standard (5 pmol each DA, 5-HIAA, DOPAC in 0.1 M perchloric acid) and fresh standards were made each day from concentrated stock. Stock solutions of 10 mM for each of DA, 5-HIAA, and DOPAC were dissolved in dH_2O and stored at 4°C. Stock solutions were made fresh each week and were diluted on the day by serial dilution in 0.1M perchloric acid.

5.2.4 Data analysis

All results were analysed using Microsoft excel and Instat2.03. Data are expressed as absolute amount of monoamine per mg wet weight of tissue and comparison of data was by ANOVA followed by multiple pair-wise comparison using Bonferroni multiple comparison test for selected pairs of columns. Statistical significance was identified as $p< 0.05$.

5.3 Results

5.3.1 5-HT and 5-HIAA concentrations in different brain regions of the mouse.

Results are expressed as pmol/mg wet weight of tissue and, where applicable, as a percentage of the value in wild-type mice.

5.3.1.1 5-HT, 5-HIAA and 5-HIAA/5-HT concentrations

The midbrain and hypothalamus had the highest 5-HT concentrations (6 pmol/mg wet weight tissue) in both wild-type and transgenic animals (Table 5.1). The midbrain area in this dissection contains the raphé nuclei and so a high proportion of serotonergic cell bodies is not unexpected. The 5-HT concentrations of the midbrain also had the highest error values of all the parameters measured. This error was probably due to differences in dissection and the possible loss of some of the raphé area as well as individual variation between animals. The high levels of 5-HT in the raphé area and hypothalamus are consistent with the published literature for both mice and primates although absolute values vary between species and investigators (Azmitia and Gannon, 1986). The order of 5-HT concentration in the other regions (highest first) was basal ganglia > hippocampus > brainstem > frontal cortex. These regions, as measured, all have a 5-HT concentration around 2-2.5 pmol/mg wet weight tissue.

The midbrain and hypothalamus also have the highest levels of 5-HIAA in proportion to their 5-HT concentrations. The ratio of 5-HIAA/5-HT in the hippocampus of transgenic mice is significantly higher ($p < 0.01$) compared to each of the other measured regions except the cortex (ANOVA followed by Dunnets pairwise comparison). There are no significant differences between any of the other regions (ANOVA followed by Bonferroni's post-hoc analysis) (Table 5.1).

	5-HT			5-HIAA			5-HIAA/5-HT		
TISSUE	**Wild-type**	**Transgenic**	**Change (%WT)**	**Wild-type**	**Transgenic**	**Change (% WT)**	**Wild-type**	**Transgenic**	**Change (%WT)**
Cortex	1.97±0.08	1.32±0.098	-33%	0.89±0.158	1.30±0.08	+42%	0.44±0.07	0.98±0.07	+121%***
Brainstem	2.33±0.17	1.59±0.098	-32%	1.22±0.083	1.38±0.14	+15%	0.52±0.02	0.87±0.06	+67%**
Midbrain	6.45±0.23	5.09±0.53	-21%***	2.71±0.2	3.05±0.57	+13%	0.43±0.02	0.68±0.2	+55%
Basal ganglia	2.54±0.17	2.18±0.09	-14%	0.96±0.08	1.16±0.06	+21%	0.38±0.01	0.53±0.02	+41%
Hippocampus	2.83±0.20	1.72±0.17	-40%***	1.98±0.16	2.12±0.24	+7%	0.72±0.07	1.23±0.09	+71%***
Hypothalamus	6.08±0.14	5.05±0.20	-17%**	3.11±0.22	3.28±0.27	+6%	0.51±0.03	0.65±0.04	+27%
Cerebellum	0.89±0.12	0.87±0.15	-2%	0.54±0.044	0.66±0.06	+21%	0.63±0.04	0.89±0.19	+24%

Table 5.1 Comparison of 5-HT and 5-HIAA concentration and 5-HT turnover in wildtype and h5-HTT mice.

Data are expressed as pmol/mg wet weight of tissue ± SEM. * indicates the significant difference between the values for transgenic and wild-type mice. *= $p<0.05$ **= $p<0.01$ ***=$p<0.001$ in ANOVA followed by Bonferroni multiple comparison test. Transgenic mice were from line A102.3 n=8. WT: wild type

5.3.2 5-HT and 5-HIAA concentrations in h5-HTT transgenic mice

Transgenic mice have reduced 5-HT concentrations in all brain areas compared to wild-type animals (Table 5.1, Figure 5.1). The decrease was 14 - 40% of wild-type values and was greatest in areas with the highest level of 5-HT turnover: hippocampus (40%) > cortex (33%) > brainstem (31%) >midbrain (21%) > hypothalamus (17%) > basal ganglia (14%). The cerebellum of transgenic animals is included as a control as it has low levels of 5-HT and 5-HTT expression, and no measurable increase in 5-HTT expression (see Chapter 4). There is no significant alteration in any parameter measured in the cerebellum.

There is a slight but non-significant increase in 5-HIAA levels in over-expressing mice, 6-42% in most areas compared to wild-type mice. (Figure 5.2).

5.3.3 5-HIAA/5-HT ratio and 5-HT turnover in h5-HTT over-expressing mice

Transgenic mice show an increase in all brain regions (27-121%) in the ratio of 5-HIAA/5-HT compared to wild-type mice (Table 5.1, Figure 5.3). Again, this increase was not significant in midbrain or cerebellum. The frontal cortex had the highest increase (121%). The hippocampus, brainstem, midbrain and basal ganglia all show changes of similar magnitude (40-50%). However, the increase (27%) in the hypothalamus was considerably lower than that in other regions and not much higher than that in the cerebellum. Increased 5-HTT expression consistently decreased total 5-HT levels and increased 5-HT turnover. The slight regional specificity of these effects may be relevant in the behavioural analysis and receptor studies.

5.3.4 DOPAC and dopamine

There was no alteration in the levels of DOPAC in mice over-expressing the 5-HTT. In one experiment there was a change in the DOPAC levels in the basal ganglia in the 5-HT assay. The dopamine levels were therefore analysed in basal ganglia

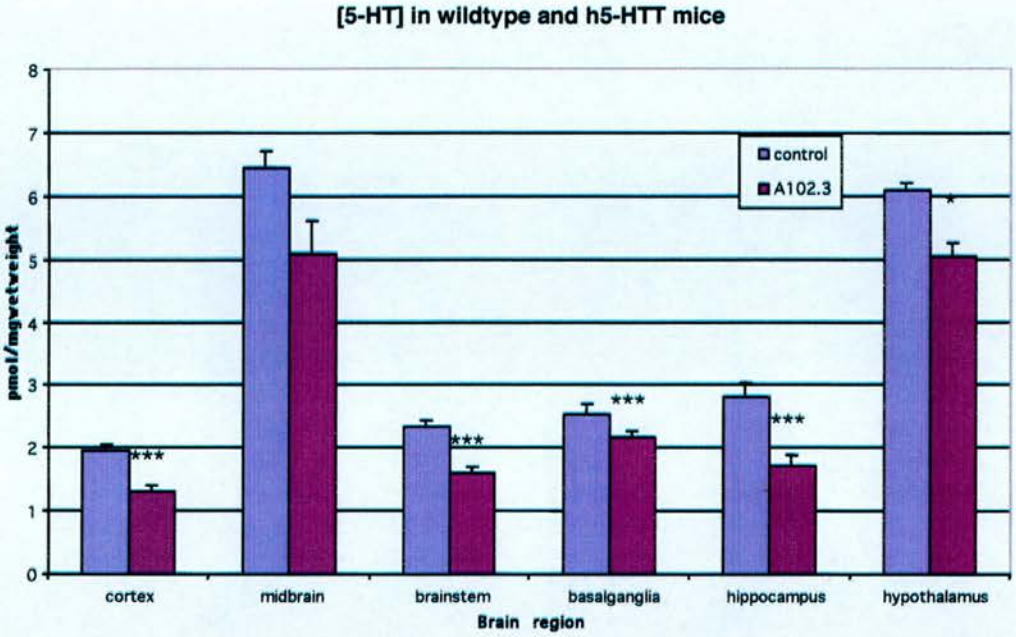

Figure 5.1 Tissue concentrations of 5-HT in different brain regions of wildtype and h5-HTT mice.

Data shown as mean± sem of 8 animals *=p<0.05 **=p<0.01 ***=p<0.001

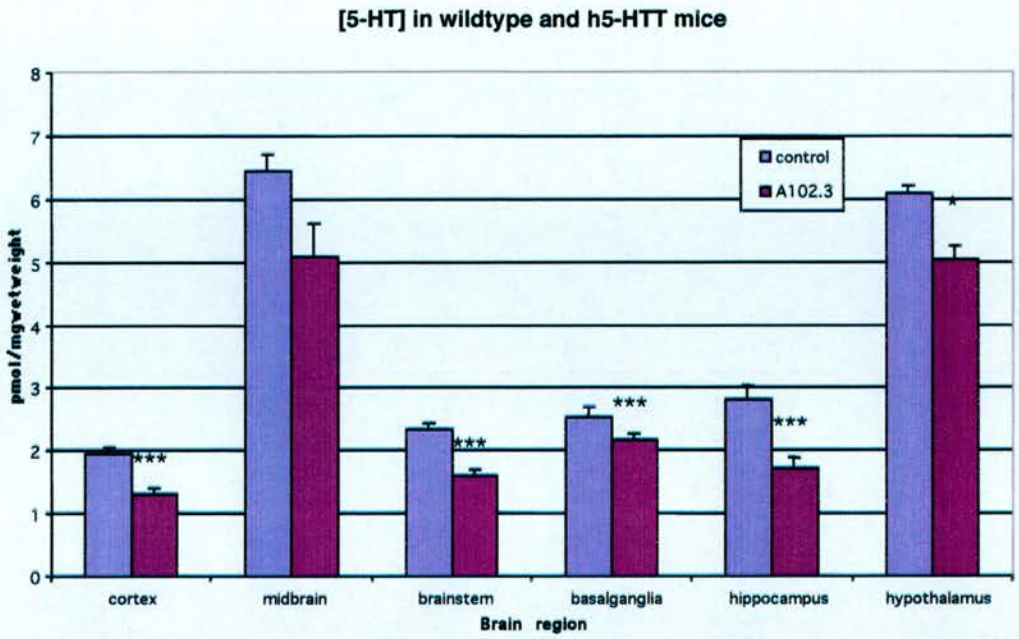

Figure 5.1 Tissue concentrations of 5-HT in different brain regions of wildtype and h5-HTT mice.

Data shown as mean± sem of 8 animals *=p<0.05 **=p<0.01 ***=p<0.001

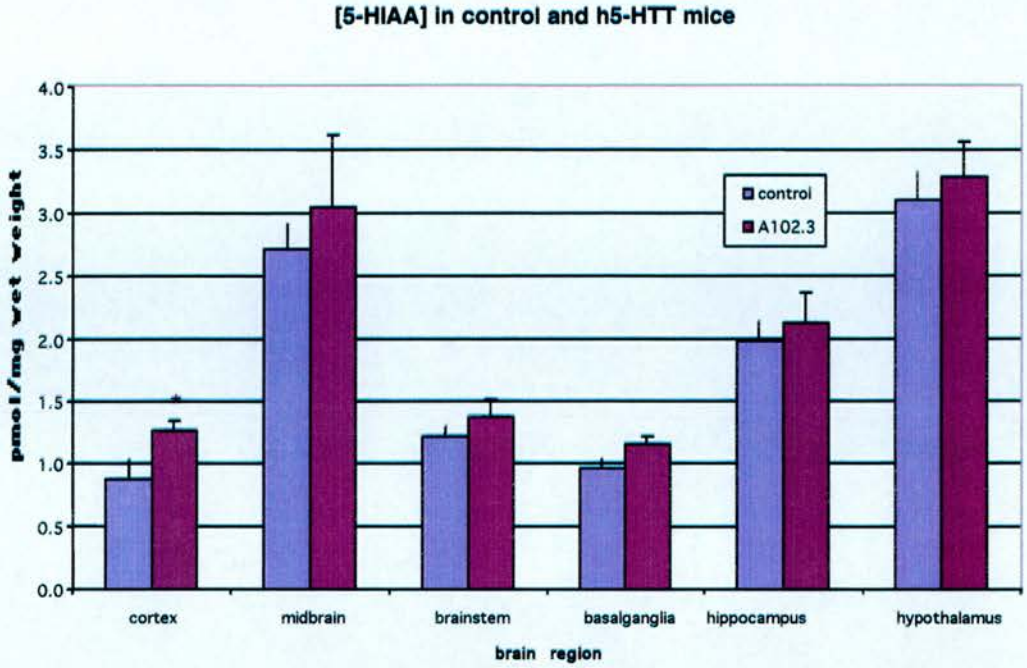

Figure 5.2 Tissue concentrations of 5HIAA in different brain regions of wildtype and h5-HTT mice

Data shown as mean± sem of 8 animals *=$p<0.05$

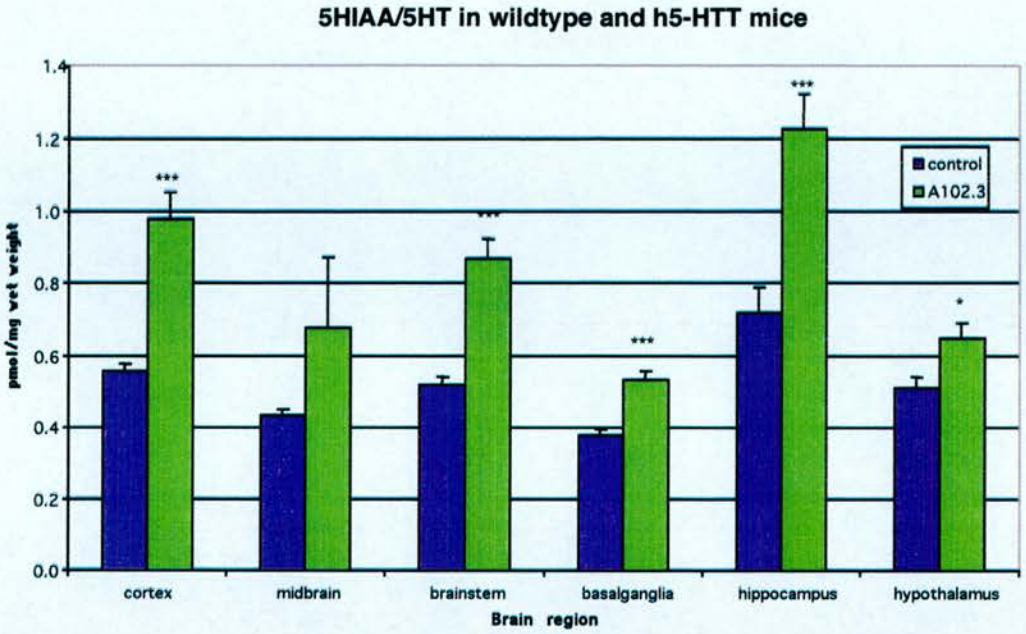

Figure 5.3 5-HT /5HIAA and 5-HT turnover in wild type and h5-HTT mice.

* p<0.05 ** p<0.01 ***p<0.001 n=8

samples. However there were no significant alterations in DA or DOPAC concentrations (Figure 5.4) or DOPAC/DA ratio and the original finding was not repeatable. Thus, it would appear that the increase in 5-HTT and decrease in 5-HT levels does not in turn alter dopamine metabolism, at least at the tissue level measured here. It is possible that alterations do occur at the synaptic level, which are not reflected in the tissue as a whole. Further investigations are needed before any definitive statement can be made.

5.3.4 DOPAC and dopamine

There was no alteration in the levels of DOPAC in mice over-expressing the 5-HTT. In one experiment there was a change in the DOPAC levels in the basal ganglia in the 5-HT assay. The dopamine levels were therefore analysed in basal ganglia samples. However there was no significant alterations in DA or DOPAC concentrations (Figure 5.4) or DOPAC/DA ratio and the original finding was not repeatable. Thus, it would appear that the increase in 5-HTT and decrease in 5-HT levels does not in turn alter dopamine metabolism, at least at the tissue level measured here. It is possible that alterations do occur at the synaptic level, which are not reflected in the tissue as a whole. Further investigations are needed before any definitive statement can be made.

5.4 Discussion

5.4.1 5-HT turnover and 5-HT concentrations

Transgenic mice contain the same levels of endogenous mouse 5-HTT as wild-type animals, with an additional over-expression of the human 5-HTT at about two times the level of the endogenous mouse form. Transgenic mice have reduced 5-HT

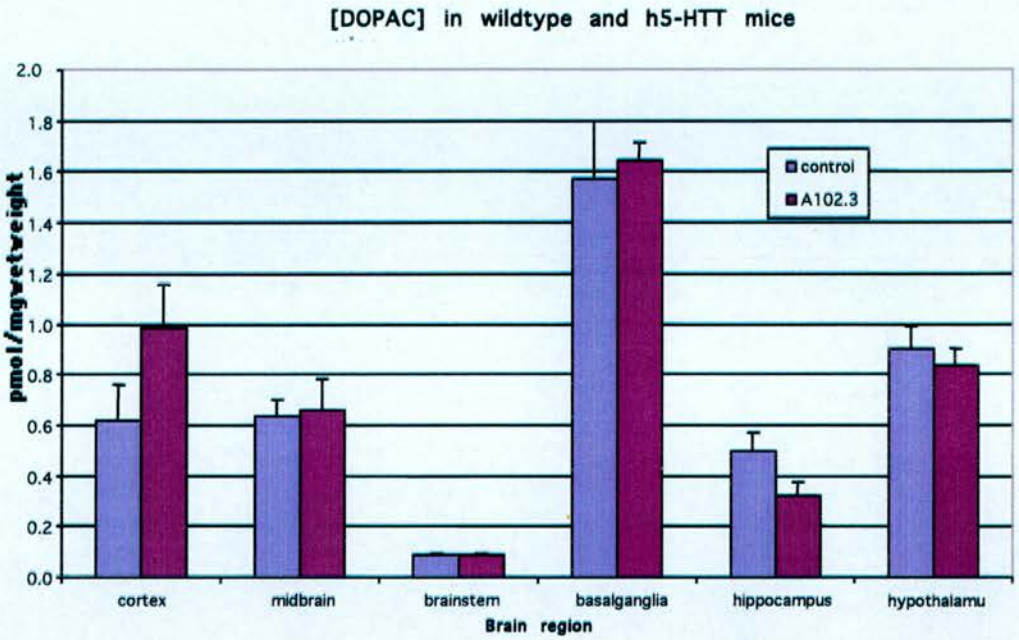

Figure 5.4 Tissue concentrations of DOPAC in different brain regions of wildtype and h5-HTT mice.

Data shown as mean± sem of 8 animals *=p<0.05 **=p<0.01 ***=p<0.001

concentrations in all tissues measured and an increase in the concentration of the metabolite 5-HIAA though these do not reach significance. The increase in 5-HIAA is not as great as the reduction in 5-HT concentrations, implying that more 5-HT is being metabolised but that stimulation of the metabolic pathway is not the only factor involved in the decline in tissue 5-HT. For the 5-HT transmitter to enter the metabolic pathway it must first be released from vesicular storage and be taken up by the 5-HT transporter. The increased metabolic product and the increased 5-HIAA/ 5-HT ratio, which is used as a measure of turnover, suggest that more 5-HT is being released from the neurons of transgenic mice than would occur in wild-type animals. Microdialysis studies of extracellular 5-HT concentrations are needed to confirm this observation. The decrease in 5-HT brain concentrations in transgenic mice (14-40% of the concentration in the corresponding area in wild-type mice) is significant in most areas, but considerably less than the 70-90% decrease seen in 5-HTT knockout mice (Bengel et al, 1998). In 5-HTT knockout mice the loss of re-uptake prevents recycling of 5-HT and so tissue 5-HT is dependent upon the synthesis of new transmitter. This mechanism can not cause the decrease in 5-HT in the animals over-expressing h5-HTT.

A decline in tissue 5-HT concentration could be due to a change in any of three general areas: increased metabolism; reduced storage; reduced synthesis; or to a combination of these factors. These points will be considered below. The 5-HT system is tightly controlled at the level of autoreceptors on both soma and terminal regions as well as more directly by 5-HT within the neuron. It is therefore probable that there are many changes which interact and can not be separated at this level of analysis. An attempt is made to consider the possible explanations and to indicate the areas most likely to be involved in these particular transgenic animals.

There are two possible states of the 5-HT system of transgenic mice: first, the status of the neurons is the same as wild-type animals except for the increase in transporter sites; second, over-expression of the transporter leads to adaptive changes in 5-HT receptors and neuronal function during development. Possible explanations for how increased 5-HTT leads to a decline in tissue 5-HT could be a direct effect of

increased uptake coupled to autoreceptor activity, altered pattern of transporter expression, adaptive changes in receptor-mediated control, and developmental changes in synthesis.

If we assume that the 5-HT system is unchanged except for the increased 5-HTT expression, then the slight increase in 5-HIAA concentrations and the increase in 5-HIAA/5-HT ratio indicates an increase in 5-HT turnover. This increased turnover suggests that greater release balances the increase in available uptake sites and that this extra release leads to a greater proportion of transmitter being broken down by MAO. This effect on metabolism can be simply explained by the increase in 5-HTT levels causing an increase in availability of uptake sites, thus increasing the rate of uptake so that more 5-HT enters the neuron in the time immediately following release. This chain of events will lead to greater intracellular 5-HT concentrations and greater activation of, and availability of substrate for, the MAO pathway. It is important to remember here that the over-expression of the 5-HTT has been present throughout life. The parameter measured is therefore the final product of all the adjustments in the system that have occurred to maintain 5-HT function at the optimum level. For transmitter release, and consequently metabolism, to be increased but synthesis reduced, an imbalance in the regulatory control must occur. Several receptor subtypes have been shown to influence 5-HT synthesis and release; both the somatic 5-HT_{1A} and terminal 5-HT_{1B} receptors act to reduce synthesis, firing and release of 5-HT. The 5-HT_{1A} receptor is probably not tonically active, so reduced extracellular 5-HT would not increase release via this receptor. The terminal 5-HT_{1B} receptor is tonically active and so altered 5-HT will affect release and synthesis via this receptor. Increased 5-HT transporter could alter synthesis through three stages. In wild-type mice 5-HT synthesis is under the control at the level of tryptophan hydroxylase activity by 5-HT_{1B}, possibly 5-HT_{1A} and end product control by intracellular 5-HT concentration (Hamon and Glowinski, 1974). When 5-HTT is increased, uptake is enhanced, the extracellular 5-HT concentration ([5-HT]) decreases and intracellular [5-HT] increases faster in the time after release. This will reduce the 5-HT_{1B} tone compared to wild-type and decrease the inhibition of release and synthesis. It may also reduce inhibition via 5-HT_{1A} or increase 5-HT_{1A}

sensitivity by preventing down-regulation. Increased intracellular [5-HT] will act to inhibit synthesis by reducing tryptophan hydroxylase activity, whilst metabolism of 5-HT via MAO to 5-HIAA is increased. The reduced inhibition leads to increase release. The increased release compensates for the augmented uptake and [5-HT] is maintained at nearer the wild-type level. The total amount of 5-HT transported into the neuron also increases, further inhibiting TPH activity. This receptor model may be all that is required to explain the altered 5-HT neurochemistry in these transgenic animals. However, it is likely that 5-HT_{1A} will be more involved. Recent evidence also indicates that 5-HT_{1B} receptors are found on the soma as well as terminal regions and that 5-HT_2 receptors found on 5-HT cell bodies in the raphé nucleus may have opposite functional effects to 5-HT_{1A} receptors, causing hyperpolarization. However, their physiological role and effectiveness has not yet been characterised. It is possible that the two opposing receptor subtypes are activated at different [5-HT] and as such the balance may be shifted in transgenic mice.

A further point to consider is that the protein expression detected by [^{3}H]citalopram binding in cortical and brainstem membranes shows a much greater proportional increase in the cortex than brainstem. Transgenic mice may therefore have an imbalance in 5-HT uptake in the terminal and cell bodies, leading to unequal stimulation of different receptor subtypes. However, in the normal mouse, there are almost certainly different excesses of transporter available in different areas of the brain , because the increase in 5-HT concentration in the raphé nuclei and ventral hippocampus reaches maximum at a lower dose of fluoxetine than that needed in the frontal cortex (Malagie et al., 1995). Thus over-expression will increase this 5-HT pool, but may not have an effect proportional to the increase. This may also help to explain why a transgenic mouse with two to three times the endogenous transporter does not show an equivalent two-thirds reduction in total tissue 5-HT. In summary, more release will occur at normal activity levels in the neuron. A greater proportion of this 5-HT than usual is broken down by MAO, as suggested by the increase in 5-HIAA concentrations, but the increased intracellular [5-HT] and lack of autoreceptor stimulation prevents a compensatory increase in synthesis and so a decline in total 5-HT concentrations in the tissue would occur.

The only brain region where the change in 5-HIAA concentration is significant is the frontal cortex. This may be due to differences in the increased expression in the different regions or to the availability of monoamine oxidase to break down 5-HT or to the availability of the enzymes and acid transport to further break down 5-HIAA in different areas. The cortex, which has the lowest 5-HT and 5-HIAA levels in wild-type animals, may have insufficient capability to remove extra 5-HIAA generated which then builds up. It may also be due to the different proportions of 5-HT which go to storage or breakdown in different areas possibly due to more expression or differences in MRN or DRN neuronal expression and innervation. The DRN and MRN have different autoreceptor reserves (Larsson et al., 1998; Malagie et al., 1996) and SSRIs have region differences in activity (Le Poul et al., 2000). The frontal cortex receives serotonergic innervation almost solely through the dorsal raphé cortical tract whilst other areas receive several serotonergic pathways.

The above explanation for the decline in tissue 5-HT concentrations assumed that there were no changes in the expression of other parts of the 5-HT system due to the presence of the transgene during development. It is not confirmed that the protein is expressed at higher than normal levels during development though the increase in adult brain would strongly suggest that it is. 5-HT is an important developmental signal and 5-HTT expression is seen both in 5-HT neurons and in non-serotonergic cells where the 5-HT that enters the cell is a trophic factor. Treatment of expectant mothers with drugs that vary 5-HT levels leads to developmental malformations. Knockout mice which have increased 5-HT levels during development, show 5-HT uptake via DA and NA transporters (Cases et al., 1998) and have altered development of the barrel field of the somatosensory cortex (Cases et al., 1996; Vitalis et al., 1998). Most knockout mice where a part of the 5-HT system is affected show adaptive changes, particularly in receptor function and expression. 5-HT_{1A} and 5-HT_{1B} receptor knockout mice have altered 5-HT and dopamine metabolism, (Ase et al., 2000) and 5-HT_{1A} receptor knockout mice have altered GABA(A) receptor expression. 5-HTT knockout mice have reduced 5-HT concentrations (Bengel, 1998) and altered 5-HT_{1A} expression and function (Fabre et al., 2000; la Cour et al., 2001;

Li et al., 1999a), 5-HT_{1B} expression (Fabre et al., 2000), and 5-HT_{2A} expression (Rioux et al., 1999) as well as altered adenosine receptor function (Mossner et al., 2000). MAO-A knockout mice have altered adenosine receptor function (Mossner et al., 2000). It is therefore probable that the 5-HTT over-expression will also cause adaptive changes in receptor function during development. These changes will probably be brain region-specific and may differentially affect 5-HT synthesis and release. It is impossible to say at this stage what changes are to be expected.

An additional facet of increased 5-HTT expression which must be mentioned is the possible change in transporter location on the neurons. Zhou *et al.* (2000) showed that the 5-HTT is found on the cell body and on axons close to the terminal bud, but probably not directly on the synaptic bud itself. Increasing 5-HTT expression may have any of four effects on positioning. Firstly, the transporters may be in the same position but very close together (a 2-3 fold increase is not extreme); this may reduce the expected effect of the increase on 5-HT levels as each receptor may inhibit the removal of 5-HT by the others. Secondly, the transporters may extend further towards the synaptic cleft, increasing the likelihood of transport and faster removal of 5-HT, thus limiting the area of diffusion and activity. Thirdly, the transporters may extend further from the site of release, removing more of the 5-HT which diffuses from the release site, limiting the area stimulated and possibly subtypes of receptor, and thus altering the regulation. Fourthly, the transporters may be located a long way from the cleft or additionally on non-neuronal cell types such as glia, thus increasing the amount of 5-HT lost from the neuron and possibly the state of the glia cells. These possibilities would provide opportunities for further study.

This model would imply greater synaptic release but the same duration of action and a reduction in total neuronal 5-HT level. Under these conditions, the autoreceptors would not be greatly altered, but it is possible that increased release would increase the concentration in the synaptic cleft at the onset of the stimulus, thus increasing the intensity of postsynaptic receptor stimulation. This stimulation, in turn, would lead to greater desensitisation/down-regulation postsynaptically and so reduce either the normal response or, depending upon the number of spare receptors in each area, the

response to an increase in prolonged 5-HT activity, such as may occur in response to external stimuli.

Another factor mentioned above is that the position of the increased protein expression may be important. If the increased 5-HTT expression has a greater proportional effect at the cell body level in the raphé, then the sensitivity of the autoreceptor may be altered. In rats there is now considerable evidence that the raphé nuclei contain a pool of extracellular 5-HT and that the somatic autoreceptors are not normally activated by baseline release but only by excessive stimulation (Adell and Artigas, 1998). Thus the removal of 5-HT by excess transporter may reduce the concentration of the extracellular pool and so increase the sensitivity of the 5-HT_{1A} receptors

5.4.2 5-HTT expression and depression

Some recent evidence suggests that SSRIs are more effective in depressed adult-onset patients with a double copy of the longer 5-HTT promoter allele, which has been linked to higher 5-HTT protein expression. Also recent work by Dahlstrom *et al.* (2000) has found that in drug-naïve adolescents and children with depression there is an increase in 5-HTT in the hypothalamic/midbrain area. Chronic SSRI treatments, which cover the delayed period of onset of therapeutic activity, have been shown to reduce transporter levels and although there is some confusion as to the mechanism, it appears that mRNA decreases (Lesch et al., 1993) so regulation of transcription is likely. This regulation may be mediated by receptor activation or intracellular mechanisms. These factors suggest that increased 5-HTT expression may be a factor in depressive illness.

The results of neurochemical analysis in 5-HTT over-expressing mice suggests that one of the ways by which higher expression of 5-HTT may pre-dispose to affective disorders is by causing a decrease in total 5-HT concentrations. This decrease could occur in three possible ways. Firstly, the decrease in 5-HT may not be the direct cause of the disorder but lead to a predisposition due to changes in the feedback of

the 5-HT system, making an external trigger factor more likely to initiate a clinical manifestation. Secondly, it is also a possible that lower 5-HT concentrations diminish available 5-HT for release upon prolonged neural stimulation. A third option may be that compensatory increases in 5-HT release inhibit or alter postsynaptic functions. A study of the receptor sensitisation and levels both post- and presynaptically, as well as microdialysis studies of 5-HT release would help clarify these questions.

If depression can result from increased expression of the 5-HTT, the therapeutic effects of SSRIs would not be due to the acute effect of augmented extracellular 5-HT but to another area of the 5-HT system which has been affected by the increase in 5-HTT. This explanation would be consistent with the delay of onset for the therapeutic actions of SSRIs and with the apparent, though not consistent, effects of 5-HT_{1A} receptor antagonists on therapeutic activity of antidepressants (Blier and Bergeron, 1998; Blier et al., 1998; Dawson et al., 2000). Whilst much more work is needed to clarify this model, it is possible that these mice will provide a suitable animal model for studies of depression in humans.

In summary, over-expression of 5-HTT decreases tissue 5-HT concentrations throughout the brain. There is a slight increase in 5-HIAA concentration and 5-HT metabolism, but the reduction in 5-HT concentration is probably mainly due to an inhibition of 5-HT synthesis, probably mediated via compensatory increases in 5-HT release and autoreceptor activity. It is possible that these mice may provide a better model for the neurochemistry of drug-naïve depressed patients than 5-HTT knockout mice.

Chapter 6

Behavioural analysis of the response of transgenic mice to MDMA

6.1 Introduction

The serotonin system has been linked to many behavioural and developmental functions, including sleep (Hilakivi, 1987; Monti and Monti, 2000), circadian rhythms (Morin, 1999), sexual function (Rampin and Giuliano, 2000; Uphouse, 2000), appetite (Leibowitz and Alexander, 1998; Meguid et al., 2000), addiction (Koob, 2000), and locomotor function (Schmidt and Jordan, 2000), as well as memory impairment (Buhot et al., 2000) and psychiatric diseases (Delgado, 2000). It was therefore expected that mice over-expressing the h5-HTT, with disturbed 5-HT neurochemistry, would have an altered behavioural phenotype. This disturbance might be robust, similar to 'the serotonin syndrome', or a less overt propensity to respond to anxiogenic or pharmacological stimuli.

6.1.1 Behavioural studies in transgenic mice

Transgenic mice provide an excellent model for studying the effects of single genes on behaviour, but there are several considerations that must be remembered when planning and analysing these experiments. The genetic modifications in transgenic animals are present throughout life (long-term), so there is always the probability that compensatory modifications occur. Such a compensation has been seen in 5-HTT knockout mice, where homozygous knockout mice develop normally despite the important role of 5-HT as a developmental signal (Lauder, 1990; Lauder et al., 2000; Lauder et al., 1983).

In addition, transgenic animals used in these experiments are the product of many generations of breeding. As caging and food supply can also change behaviours, all the animals used were from cages with more than one animal, in case solitary

housing has any affect on 5-HT function. A preliminary screen (Irwin, 1968), carried out to check for any major behavioural abnormality, detected no obvious change in phenotype in 5-HTT transgenic mice. In 5-HTT knockout animals, which have been studied as a model of disturbed 5-HT function, no obvious changes in behavioural phenotype have been reported, although the locomotor effect of MDMA was lost (Bengel, 1998). Therefore, the effect of MDMA on temperature and locomotor activity was studied in the transgenic animals as a comparison with the knockout mice and to probe the effects of 5-HTT over-expression on the 5-HT system.

6.1.2 5-HT and thermoregulation in mice

The hypothalamus is the major centre for temperature regulation in the majority of mammals studied (Arancibia et al., 1996; Bligh and Hensel, 1974; Feldberg, 1969; Van Tienhoven et al., 1979). 5-HT is a major putative transmitter system in temperature regulation as intra-cerebroventricular injections of 5-HT cause hypothermia in some models (de Roij et al., 1979; Yamada et al., 1988; Yamada et al., 1987) and 5-HT concentrations vary with body temperature during heating and cooling in primates (Myers and Beleslin, 1971; Myers and Waller, 1975) and rats (Lin, 1978). There is conflicting evidence for the roles of DA, NA, acetylcholine (ACh) and 5-HT in thermoregulation, with different investigators and methods producing apparently contradictory results. For instance, intracranial injections of 5-HT have produced both a rise and fall in core body temperature (Cox et al., 1980; Crawshaw, 1972; Lin et al., 1981). Sheard and Aghajanian (Sheard and Aghajanian, 1967) found that intra-hypothalamic injections of 5-HT or electrical stimulation of the dorsal raphé increased colonic temperature in the rat, whilst Lin et al. (1983) found that electrical stimulation of the dorsal raphé or intra-hypothalamic injections lowered colonic temperature. However, a wide range of doses and methods were used in these studies. A high dose of 5-HT can cause a sharp fall in temperature (de Roij et al., 1979), perhaps because of activation of presynaptic 5-HT_{1A} receptors which leads to a decrease in neuronal firing. 5-HT_{1A} receptor stimulation by 8-OH-DPAT has long been known to cause hypothermia and it is often used as a measure

of 5-HT_{1A} activity (Goodwin et al., 1985; Hjorth, 1985). In addition to saturating 5-HT receptor sites, high doses of 5-HT can also interact with the catecholamine receptors that mediate the peripheral heat loss pathway (Ruwe and Myers, 1982).

More recent evidence from microdialysis experiments (Lin et al., 1998) using different pharmacological tools to increase or decrease 5-HT in the hypothalamus found that drugs which increased extracellular 5-HT (fluoxetine, 5-HTTP, and potassium) caused hyperthermia (measured as core body temperature) and increased metabolic rate. Drugs that reduced 5-HT levels, such as the 5-HT_{1A} agonist 8-OH-DPAT, caused hypothermia. This hypothermia, induced by reduced 5-HT in the extracellular compartment, was brought about by a decrease in the metabolic rate and an increase in skin temperature. The alteration in 5-HT level was parallel to, but in advance of, the change in temperature. DOI, a 5-$HT_{2A/C}$ receptor agonist, also induced hyperthermia. The hyperthermic effect of DOI is mediated via 5-HT_{2A} stimulation (Salmi and Ahlenius, 1998) and is enhanced by 5-HT_{1A} antagonists, suggesting a functional interaction between the two receptors and the possible involvement of the 5-HT_{1A} receptor under normal conditions. In general, 5-HT_{1A} receptor stimulation causes hypothermia and 5-HT_{2A} receptor stimulation causes hyperthermia (Gudelsky et al., 1986; Nash et al., 1989).

Changes in body temperature induced by changes in the 5-HT system can therefore be mediated via altered central 5-HT receptor balance, as a result of either increased 5-HT release activating different receptor subtypes or changes in receptor expression. In addition to its central actions, 5-HT can cause hypothermia via stimulation of peripheral 5-HT_2 receptors (Sugimoto et al., 1991). The hypothermia was prevented by ketanserin, a 5-HT_2 antagonist with greatest selectivity for 5-HT_{2A} receptors, and so is probably acting via 5-HT_{2A} receptors in the vasculature, possibly to increase heat loss.

6.1.3 5-HT and dopamine interactions in thermoregulation in mice

The dopamine (DA) system has also been implicated in temperature changes in mice and rats (Cox et al., 1980; Yamada et al., 1988). The hypothermia induced by stimulation of 5-HT_{1A} receptors is inhibited by dopamine receptor antagonists (Yamada et al., 1988), suggesting that dopaminergic systems are involved in 5-HT-induced hypothermia in mice. Dopamine D1 and D2 receptors mediate different effects on temperature in mice; D1 receptors mediate hyperthermic and D2 receptors hypothermic responses (Costentin et al., 1990; Salmi, 1998; Salmi et al., 1993; Sanchez, 1989; Zarrindast and Tabatabai, 1992). Dopamine D2/D3 receptor agonists induce hypothermia in mice (Salmi, 1998). This effect that almost certainly occurs via D2 receptor stimulation as D2 receptor knockout mice do not show either the hypothermic or hypo-locomotor effects seen in wild-type animals (Boulay et al., 1999a), whilst D3 knockout animals have similar responses as wild-type mice (Boulay et al., 1999b). D2 antagonists such as halperidol prevent the hypothermia induced by non-selective dopamine agonists, whilst D1 receptor antagonists do not. The loss of both locomotor and temperature effects of MDMA in knockout mice implies that the same neurotransmitter system may be involved in both and that the hypothermia may be in part because of reduced locomotor activity. D1 receptor stimulation reduces the hypothermia caused by dopamine released by reserpine, and D1 agonists induce hyperthermia (Nunes et al., 1991; Vasse et al., 1990; Zarrindast and Tabatabai, 1992). The hyperthermic effect is masked when D2 receptors are also stimulated (Costentin et al., 1990). The hypothermia induced by MDMA may therefore be partly mediated via changes in dopamine receptor activation or balance of the receptor subtypes as well as direct effects on dopamine release induced by 5-HT receptor activation.

5-HT_{2A}, 5-HT_{2B}, 5-HT_{1A} and 5-HT_{1B} receptors have been implicated in the control of dopamine neurons *in vivo*. Both 5-HT_{2A} and 5-HT_{2B} receptors tonically inhibit dopamine release in rats (Di Matteo et al., 1998; Millan et al., 1998). 5-HT_{1B} receptors mediate the locomotor effects of MDMA, probably via activation of DA neurons. 5-HT_{1B} knockout mice have attenuated locomotor response to MDMA

(Scearce-Levie et al., 1999) and decreased DA levels resulting from increased DA turnover (Ase et al., 2000). 5-HT_{1A} receptor knockout mice have augmented 5-HT release and turnover and show reduced inhibition of DA neurons (Ase et al., 2000). Any changes in 5-HT receptor expression resulting from adaptive changes in transgenic mice may therefore affect both locomotor and temperature effects via the dopaminergic system.

6.1.4 Role of other transmitter systems and of corticosterone in thermoregulation

Noradrenaline has also been implicated in hypothermic responses in mice (Leroux-Nicollet et al., 1988) and primates (Myers and Waller, 1975). The primate study also found that the 5-HT-induced hyperthermia was concurrent with an increase in ACh, suggesting that 5-HT activates a cholinergic pathway which transmits efferent signals for heat production. Recent evidence that MDMA alters ACh release via histamine receptors (Fischer et al., 2000) may therefore be a factor in the response to MDMA in transgenic mice.

Corticosterone reduces the hypothermia induced by 8-OH-DPAT stimulation of 5-HT_{1A} receptors, probably via direct attenuation of 5-HT_{1A} function (McAllister-Williams et al., 2001). This is probably of less importance than the major transmitter systems, 5-HT and dopamine, is mentioned for the sake of completeness.

6.1.5 Responses of mice to MDMA exposure

MDMA in mice causes an acute release of 5-HT and DA from nerve terminals. This release of 5-HT has both carrier-mediated (imiprimine-sensitive) (Gu and Azmitia, 1993; Gudelsky and Nash, 1996) and Ca^{2+}-dependent components (Crespi et al., 1997). Fluoxetine abolishes 5-HT release and attenuates DA release. MDMA causes an increase in locomotor activity, decreases exploratory behaviour and disrupts startle response. In 5-HTT knockout mice, the increase in locomotor activity evoked by MDMA is abolished, and a brief paradoxical reduction in activity occurs after MDMA administration. These mice have a pronounced reduction in 5-HT levels throughout the brain (see Introduction) that may cause this change in

response. However, the heterozygous mice have similar 5-HT levels to wild-type animals, but show a 50% reduction in locomotor activity that correlates with the level of transporter expression (Bengel, 1998). 5-HT_{1B} knockout mice also have abolished locomotor response to MDMA (Scearce-Levie et al., 1999), probably as a result of lack of stimulation of DA release in the striatum.

In addition to MDMA's locomotor effects, considerable evidence indicates that MDMA alters core body temperature in rodents, though few studies have detailed these effects in mice. Treatment regimes where multiple doses of MDMA are given to mice cause both hypothermia and hyperthermia, depending upon dose and time after injection. Multiple doses (4 repeated doses of 20 $mg.kg^{-1}$ at 2 hourly intervals) caused a short-term hyperthermia in mice (Miller, 1994; O'Callaghan, 1994), which returned to baseline after the last dose. The same dose (20 $mg.kg^{-1}$) given 3 times at 3 hourly intervals caused a brief hypothermia after the first dose, followed by hyperthermia after the second dose which was maintained for several hours (until recording stopped) (O'Shea, 2001). At a higher dosing regime (3 doses of 30 $mg.kg^{-1}$ at 3 hourly intervals), a short-term hyperthermia occurred after the first dose, followed by hypothermia an hour after the last dose. A lower dose (10 $mg.kg^{-1}$) caused only hypothermia for the first 2.5 hours and no further change (O'Shea, 2001). This study suggests a biphasic, dose-dependent effect of MDMA on body temperature. Although effects of MDMA in these studies were described as prolonged and long-term, there were no studies of 24-hour temperature measurements in mice after single doses of MDMA. With multi-dose regimes it is difficult to ascertain the concentration of MDMA in the plasma or at the site of action or to control the secondary effects of reduced 5-HT availability. At low doses, 5-HT release may be sub-maximal (such that only a proportion of 5-HT stores is released). A second dose at the lower concentration then has a greater effect than repeated dosing at a higher dose where 5-HT stores may be depleted more quickly. The one study that used radio-telemetric methods to monitor body temperature over a long period (Dafters R.I, 1998) found that 4 single daily doses of MDMA given to rats on consecutive days produced a thermogenic response that depended on ambient temperature and caused long-lasting alterations in thermoregulatory mechanisms.

MDMA use in humans is known to produce hyperthermia that, combined with dehydration, is often considered to be one of the possible causes of ecstasy-related deaths (Burgess et al., 2000; Dar and McBrien, 1996; Mallick and Bodenham, 1997). Considerable evidence from studies on rats suggests that preventing MDMA-induced hyperthermia by restraint or pharmacological means reduces 5-HT neuronal toxicity (Broening et al., 1995; Colado et al., 1998; Colado et al., 1993; Malberg et al., 1996; Sabol and Seiden, 1998). Several studies, however, suggest that there is another pharmacological, but non-temperature-sensitive component (Colado et al., 1998; Malberg et al., 1996). In mice there is no 5-HT nerve toxicity at low doses of MDMA though there is striatal DA neuronal toxicity (Logan, 1988; Stone, 1987). There is some evidence that reducing body temperature, either by pharmacological means (MK-801 or ethanol) or restraint, prevents DA toxicity (Miller, 1994). However, another study found that pretreatment with fluoxetine to prevent the MDMA-induced temperature changes did not prevent toxicity, whilst the dopamine reuptake inhibitor GBR 12909 was neuroprotective but did not prevent temperature change (O'Shea, 2001). MDMA causes long-term neurotoxicity to the 5-HT system in humans and primates (see introduction).

The aim of the studies described in this chapter was to provide a preliminary characterisation of the possible effects of increased 5-HTT expression on the behavioural phenotype of transgenic mice and their behavioural responses to pharmacological challenge. MDMA was used to probe the 5-HTT system's activity in the transgenic mice in order to determine whether increased 5-HTT levels, accompanied by moderately reduced 5-HT levels, alter locomotor behaviour. The effects of increasing doses of MDMA on core temperature in the mouse were also investigated. A dosage regime was chosen to include the range of doses used in the literature to induce maximal locomotor activity in mice (Bengel, 1998; Scearce-Levie, 1999) as well as a lower range in case the over-expression of the 5-HTT sites increased sensitivity to MDMA. The use of radio-telemetry implants rather than rectal temperature probes allowed the 24-hour monitoring of both locomotor activity

and temperature. The neurotoxicity of MDMA in mice is dose- and frequency-dependent (O'Shea et al., 1998).

We predicted that over-expression of the human 5-HTT in the transgenic mice might lead to 5-HT nerve terminal loss and long-term 5-HT disturbance. This possibility was considered as it is known that 5-HT and 5-HIAA levels return to baseline after 24 hours in wild-type mice (Logan, 1988) and so disturbance after this washout period would indicate a neurotoxic action. Mice were left for 72 hours after the last dose of MDMA before tissue samples were taken for HPLC analysis. Because the number of animals tested was small, only preliminary telemetry data are presented.

6.2 Methods

6.2.1 Irwin Screen

The Irwin screen is a comprehensive assessment of the behavioural, neurological, and autonomic state of mice, allowing one to distinguish the effects of psycho-active, neurologic and autonomic drugs between different strains of mice, and the different effects of drugs in the same class (Irwin, 1968). This screen was used as a basic test of the phenotype of transgenic mice. This screening process is most sensitive at detecting behavioural changes induced by pharmacological challenges. However, it can also measure behaviour related to arousal and anxiety and has been used to detect differences between strains of mice. It is in some senses a very crude system, but covers a broad range of behaviours and should pick up any major behavioural differences between transgenic and wild-type mice.

The complete method has been previously described (Irwin, 1968). Briefly, six to eight second-generation transgenic +/- males from lines A102.3 and A145.1 (one line using each YAC construct and both with high expression of 5-HTT RNA) were compared to non-transgenic littermates and, where littermates were not available, to second-generation wild-type mice to provide a similar background. Tests were done in a separate room from the home room by a trained observer who was blind to the

genetic status and line of the animals tested. Mice were tested with a series of simple behavioural paradigms to measure: spontaneous activity, motor-affective response, sensori-motor response, posture, muscle tone, equilibrium and gait, CNS excitation, appearance of eyes, secretion and excretion, and general behaviour. The order in which tests were carried out minimised the handling stress and the more provocative tests were done last.

It had been observed during routine handling that transgenic mice had a tendency to be smaller and more resistant to handling. We considered the possibility that mice over-expressing the 5-HTT would have altered 5-HT receptor expression that might lead to increased anxiety or aggression. In this case a change in the motor-affective responses or startle response would be expected. These responses cover general state, responses to new environments, stimuli, and aggressive challenge. For example, vocalization and urination-defecation are responses which give information on general state; transfer arousal and alley progression give information on response to new environment; and provoked biting and grasp-irritability on aggressive impulses.

Analysis was by ANOVA followed by Dunnett post-test's analysis of each transgenic line to the wild-type where such tests were possible (see results).

6.2.2 Telemetry apparatus

The freely moving telemetry system (Data Sciences International) uses a transmitter and monitor pill inserted into the peritoneal cavity. The monitor sends a signal to a receiver attached to a data analysis system (Data Quest) that stores the data. The monitor used here measured body temperature and horizontal locomotion. Telemetry provided a convenient way to measure activity and temperature without the need for restraint to insert rectal temperature probes. The monitors also measure horizontal rather than running-wheel activity, which is important as MDMA-induced locomotor activity tends towards an increasing straight line pattern of locomotion (Gold et al., 1988), accompanied by reduced exploratory behaviour and low pattern

variability. Telemetry monitoring also allows 24-hour continuous measurement, which is important in the long-term measurement of temperature, and which provides a measure of circadian variations as it covers both the light and dark periods.

6.2.3 Dosing regime

Transgenic heterozygous males, 4-6 months old, were compared to their non-transgenic littermates. Animals were treated with MDMA or saline, in groups of eight, consisting of a random mixture of wild-type and transgenic animals. To measure body temperature and horizontal locomotor activity, telemetry pills (PhysioTel Implants, Data Sciences International) were surgically implanted into the peritoneal cavity under general anaesthesia and the animals allowed to recover for 3-4 days before being placed in the experimental homecage. Mice were individually housed in polycarbonate cages (Eurostandard Type 2L, 365 x 207 x 140 mm), each equipped with a running wheel (240 x 80 mm from Techniplast UK Ltd, UK). Ambient temperature was maintained at 22°C. Deep litter bedding (40 mm depth) was provided in each cage; food and water were available *ad libitum*. Running-wheel activity was monitored by means of reed relays, magnetically sensitive switches, activated by four miniature magnets positioned 90° apart on each wheel. One count was equivalent to 189 mm traveled horizontally. The detectors were monitored continuously by Dataquest ART system (DSI, MN, USA) that collated activity counts into ten-minute time bins across the duration of the experiment. Animals were allowed to acclimatise to the experimental environment for 1 week (12 hr.-12 hr. light/dark cycle, 7.00am/7.00pm). A total of 7-8 age-matched animals for each genetic background were treated either with saline or MDMA (one animal died after implantation of the monitor pill).

Animals were allowed to adjust to the homecage for 24 hours for baseline recording. On the second day the animals were injected with MDMA or saline at hour 4 of the circadian cycle (11am). MDMA was dissolved in 0.9% saline and doses were given subcutaneously in a ramped regime: on day two, $1mg.kg^{-1;}$ day three, $3mg.kg^{-1}$; day four, $10mg.kg^{-1}$; and day five, $30mg.kg^{-1}$. Monitoring was maintained throughout.

Animals were then monitored for another 72 hours before culling. This range of doses was chosen to cover the doses in the literature which gave maximal locomotor effects in different strains of mice (Bengel, 1998; Scearce-Levie, 1999) and to see whether transgenic animals responded at lower doses. As mentioned in the introduction , compared to humans and rats , mice are resistant to the toxic effects of chronic MDMA treatments. The presence of the human transporter in the transgenic mice may increase their susceptibility to the toxic effects of high doses of MDMA. The mice were culled 72 hours after the last dose and it was not possible to measure any parameters of neuronal cell death. This must be kept in mind we analyzing the results from the later doses. However it is possible that the resistance to MDMA is due to another variable in the mouse neuron and is not related to the 5-HTT. The short time between doses and the lack of any residual effects of the MDMA at 72 hrs post treatment suggests that neuronal toxicity is probably minimal under this regime.

Statistical analysis was by two-way ANOVA or least squares, followed by Student-Newman-Keuls test.

6.3 Results

The data presented are a preliminary analyses of temperature and horizontal activity. Statistical analysis with thanks to Dr Hugh Marston, FINE, Edinburgh.

6.3.1 Irwin screen

The results of behavioural observations, grouped according to the key factors they provide information about, are shown in Table 6.3.1. As the screening process covers many variables which can be seen in different mouse lines, some variables, such as eye colour, were not appropriate for this line of mice and are therefore not listed in the results.

This form of analysis was used as a preliminary screen to catch any gross phenotype and to suggest areas for further study. As many of the variables are scored on an

		Wild-type	102.3	145.1
Spontaneous activity	locomotor activity	3.7	3.5	4.1
	bizarre behaviour	0	0	0
Motor- affective response	alley progression	37	63.5	48
	transfer arousal	4	4	4
	touch- escape	5.3	7	4.9
	posit. struggle	3.3	4.25	1.38
	grasp-irritability	3.2	3.38	1.25
	provoked biting	1.3	4.29	3.75
	provoked freezing	0.2	0	0
	finger withdrawal	0.2	0.25	1.4
	finger approach	1.3	1.4	1.4
	posit.passivity	0.2	0.25	0.13
	vocalization	2.33	3.13	3.38
	urination-defecation	0.5	0.63	0.38
Sensoro-motor response	visual placing	7	6	4.5
	tail-pinch	3.3	4.9	4
	toepinch	6	5.9	5.6
	corneal	4.7	4.25	4
	pinna	5.8	6.5	5.1
	startle	3.8	3.8	2.8
Posture	pelvic elevation	4	4	4
	tail elevation	1.8	2.25	2
	limb rotation	0	0	0
Muscle tone	body tone	5.2	5.3	5
	abdominal tone	4.7	6.1	4.6
	limb tone	3.8	4.1	3.5
	grip strength	5	4.9	5.8
	wire manoeuvre	1.5	1.5	0
Equilibrium and gate	righting reflex	0	0	0
	ataxic gate	0	0	0
	hypotonic gate	0	0	0
	gate other	–	–	–
	total incapacity	0	0	0
Secret. & excret.	salivation	0	0	0
	lacrimation	0	0	0
	diarrhoea	0	0	0
General	hypothermia	0	0	0
	piloerection	0	0	0
	skin colour	–	–	–
	respiratory rate	3.7	3.75	4

Table 6.3.1 Results of Irwin screen in transgenic and wild-type mice. Results are expressed as the mean of 6-8 animals .102.3 & 145.1= transgenic line A102.3 and A145.1

arbitrary, non-continuous scale, it provides a largely quantitative rather than qualitative analysis. It is also a characteristic of this sort of analysis that some of the measurements, such as alley progression, have high potential for inconsistent results.

Results are therefore best analysed by comparing all measured behaviours which would be consistent with a particular system or area being affected by the transgene. Neither line of transgenic mice had an overt behavioural phenotype when compared to wild-type animals. Nor was there a difference when both transgenic lines were combined. These measurements were all taken during the light period of the diurnal cycle and do not cover the onset or intensity of normal locomotor activity during the dark period.

6.3.2 Baseline activity and temperature in transgenic and wild-type mice

Transgenic mice from line A102.3 were compared to wild-type animals. Line A145.1 was excluded because of space limits. The telemetry apparatus was used to measure both temperature and locomotor activity throughout the 24-hour period. Baseline conditions were measured on day one in the absence of handling or injection. Data were sampled automatically with one measurement taken every 10 minutes. This frequency provided too much data to be easily analysed, therefore the measurements were pooled first as 30-minute then as 2-hour periods after injection. This time frame fitted the response profile.

Figures 6.2 and 6.3 show baseline measurements for wild-type and transgenic mice. There are two groups of each, which were later treated either with saline or MDMA, but here are drug-naïve. Wild-type and transgenic mice do not have significantly different body temperatures under baseline conditions ($p<0.05$). Transgenic animals show similar activity to wild-type animals during the light period but are less active than wild-types during the circadian night.

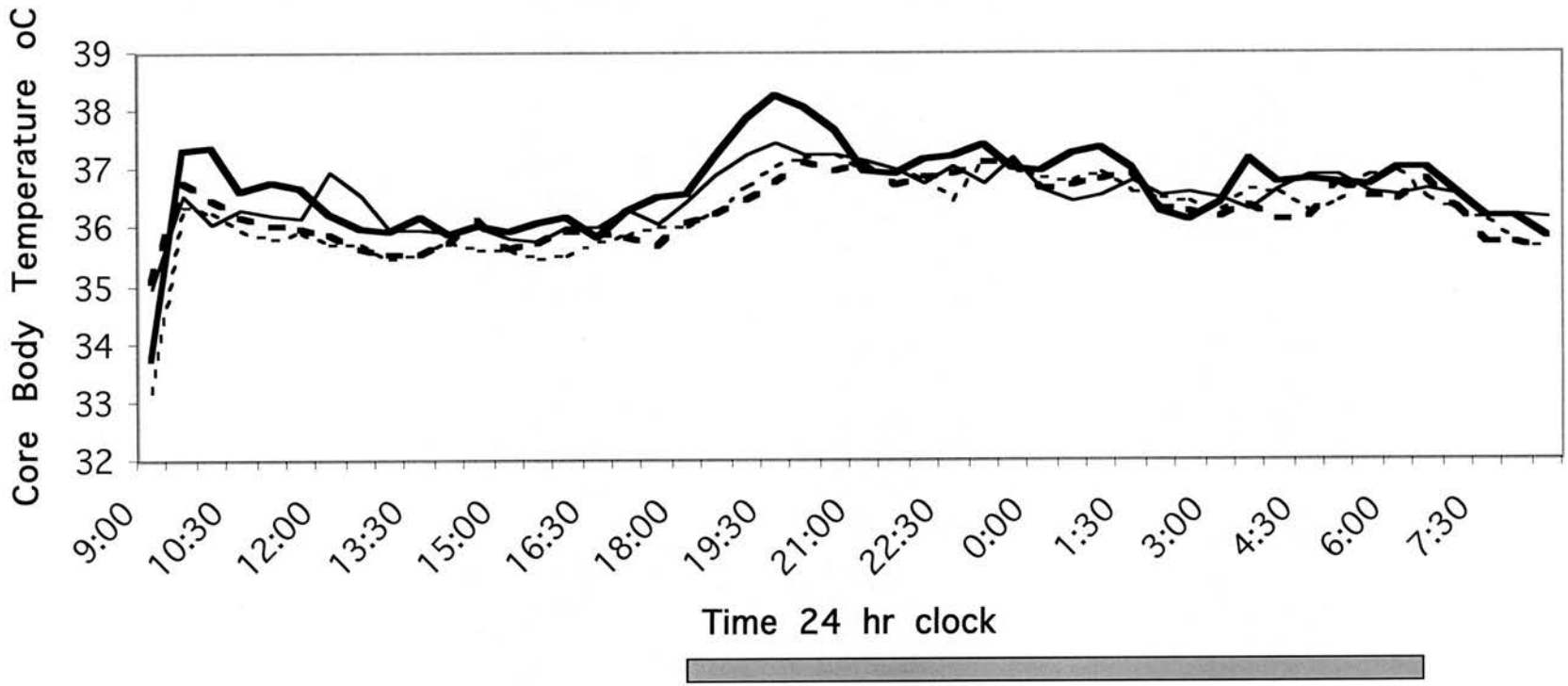

Figure 6.2 Diurnal core body temperature (^{o}C) in untreated wildtype and transgenic mice

Data is expressed as pools of 30 minutes of recordings for each animal. Each value represents the mean of 6-8 animals and 3 measurements for each animal. The dark period is indicated by the grey bar. Throughout the experiment injections were given at 10am (third circadian hour)

Groups: ——— =nontransgenic saline, ——— =nontransgenic MDMA , - - - - =transgenic saline ▪ ▪ ▪ ▪ ▪ =transgenic MDMA .

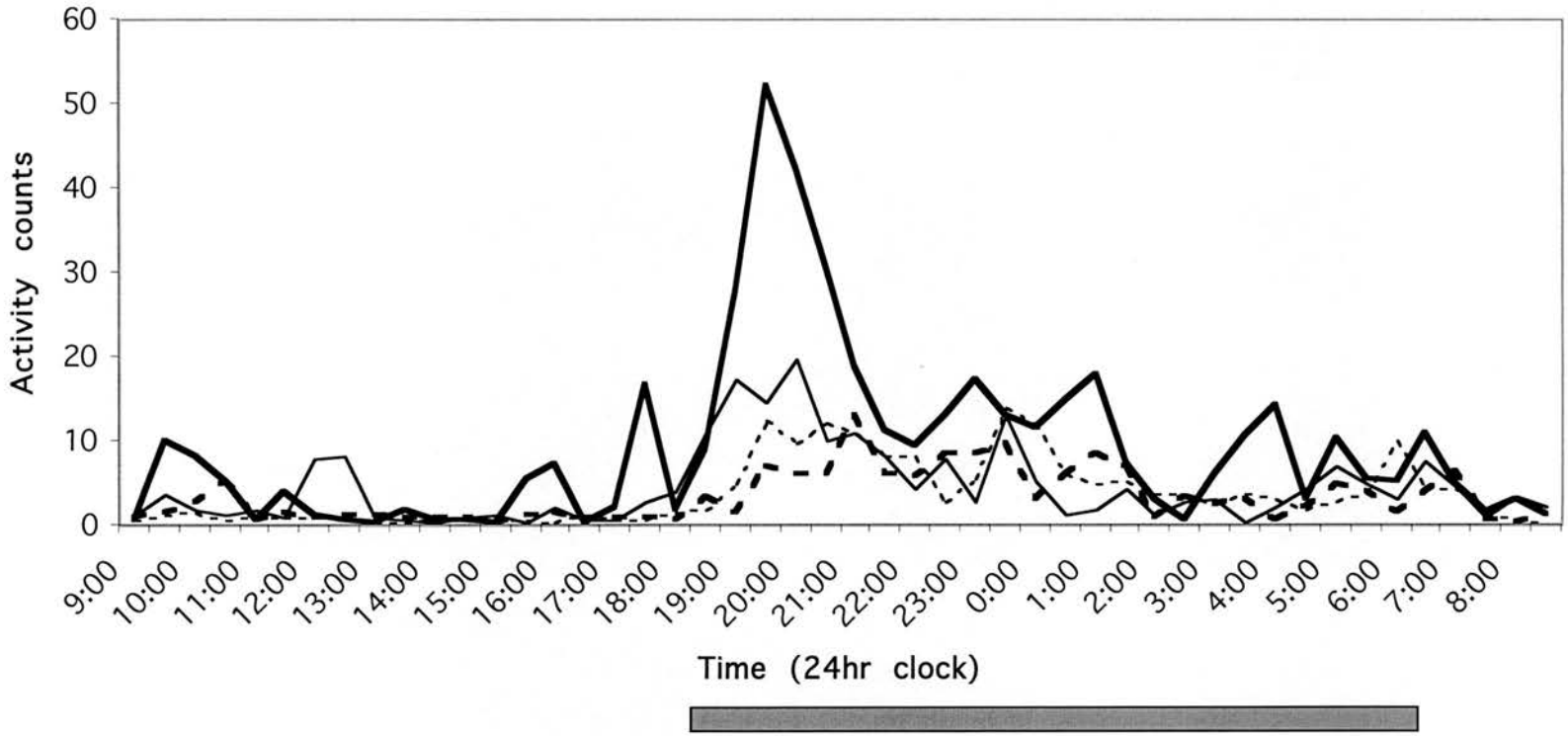

Figure 6.3 Baseline horizontal activity in untreated wild type and transgenic mice

Data is expressed as the mean of 6-8 animals and as the mean of 30 minutes of measurements for each animal (measurements sampled every 10 minutes).The graph covers the 24 hr period9am-9am , the darkperiod is indicated by the grey bar

Groups: _____ =nontransgenic saline, ___ =nontransgenic MDMA , - - - - =transgenic saline - - - - - =transgenic MDMA .

6.3.3 Effect of MDMA on temperature in wild-type and transgenic mice

Data were pooled before graphing as follows. First the results, which were absolute values without subtracting baseline (recorded on day one), were pooled into groups of recordings from each 30-minute period. The next stage of analysis was to calculate the mean of measurements for each animal during the 30-minute or 2-hour window. Finally, the mean of all animals in each group was calculated for each time period. This is the result shown on the graphs.

At the two lowest doses of MDMA there was no significant alteration in the body temperature of transgenic mice or wild-type mice. Statistical analysis of temperature differences was by two-way ANOVA of the minimum temperature attained in the two hours following MDMA administration at the two highest doses (10 $mg.kg^{-1}$ and 30 $mg.kg^{-1}$) which confirmed that there were significant factors of genotype ($p=0.004$) and drug treatment ($p=0.039$) on temperature and an interaction between the two factors ($p=0.013$). This analysis was followed by a Student-Newman-Keuls pair-wise multiple comparison to isolate the groups that differed.

	Saline	MDMA
Wild-type	35.1±0.8	35.5±0.6
Transgenic	34.8±0.6	31.5±0.6*†

Table 6.3.2 Minimum core body temperature (°C) attained by transgenic and wild-type mice in the two hours after injection with MDMA (pooled 10 and 30 $mg.kg^{-1}$ n=8). *Significantly different from MDMA-treated wild-type, $p<0.001$; †Significantly different from saline, $p<0.001$

There were no significant differences in body temperature between untreated wild-type and untreated transgenic animals (Table 6.3.2). MDMA treatment did not significantly alter the core body temperature of wild-type mice. Transgenic mice showed a significant decrease in body temperature in response to MDMA ($p<0.001$) compared to saline-treated controls. The response to MDMA was significantly different ($p<0.001$) between wild-type and transgenic mice. Thus MDMA lowered

the temperature of transgenic mice compared to the wild-type and non-treated transgenic mice.

The shape of the thermoregulatory response is shown in Figure 6.4. In both wild-type and transgenic animals MDMA treatment (10 $mg.kg^{-1}$) led to a slight (non-significant) hyperthermic response of 1-2°C following a brief hypothermia in the first hour after injection. This transient hyperthermia was probably because of increased locomotor activity and was followed only in the transgenic mice by prolonged and increasing hypothermia, which started 4 hours after injection and reached up to -5°C 6.5 hours after injection. The temperature then slowly increased and returned to baseline by 16 hours post-injection.

At a dose of 30 $mg.kg^{-1}$ in wild-type and transgenic mice (excluding one mouse with a prolonged hypothermia), MDMA evoked a slight hypothermic response for the first hour after injection, which was quickly reversed (Figure 6.5). A slight hyperthermic response of + 2°C occurred, which peaked at 4.5 hours and was maintained until 7 hours after injection and the onset of activity during the dark period. The data include one transgenic animal that did not show a hypothermic response at 10 $mg.kg^{-1}$, but did respond at 30 $mg.kg^{-1}$. In animals which responded at the high dose the hypothermic response was similar, in onset and duration, to that seen at the lower dose (10 $mg.kg^{-1}$). Data were pooled before graphing, as described for the temperature measurements above.

6.3.4 Effect of MDMA on locomotor activity in wild-type and transgenic mice

Both transgenic and wild-type mice start exhibiting locomotor responses to MDMA at 10 $mg.kg^{-1}$ ($p<0.05$). The duration of the locomotor effects over a 24-hour period are shown in Figures 6.6 and 6.7. In wild-type and transgenic animals, MDMA at 10 $mg.kg^{-1}$ led to an increase in horizontal activity after injection, which declined after the first 4 hours but remained slightly greater than baseline. This decline lasted until

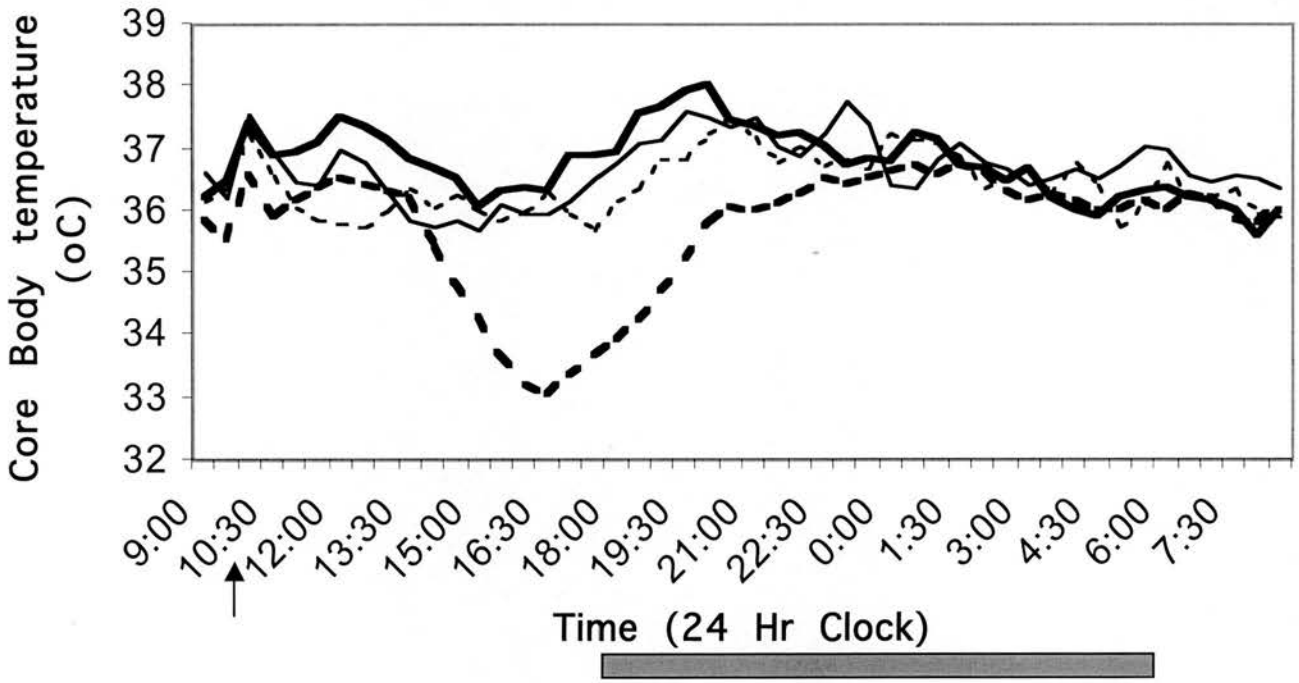

Figure 6.4 Time course of the effect of a single dose of 10mg.kg-1 MDMA on core body temperature in transgenic and wildtype mice.
Data is expressed as pooled data for each 30minute period after injection and the means of 6-8 animals .
Groups: ——— =nontransgenic saline, ——— =nontransgenic MDMA , - - - - =transgenic saline - - - - - =transgenic MDMA
The injection was given intraperitoneal (arrow) at 10am .The graph covers the 24 hr period9am-9am , the dark period is indicated by the grey bar

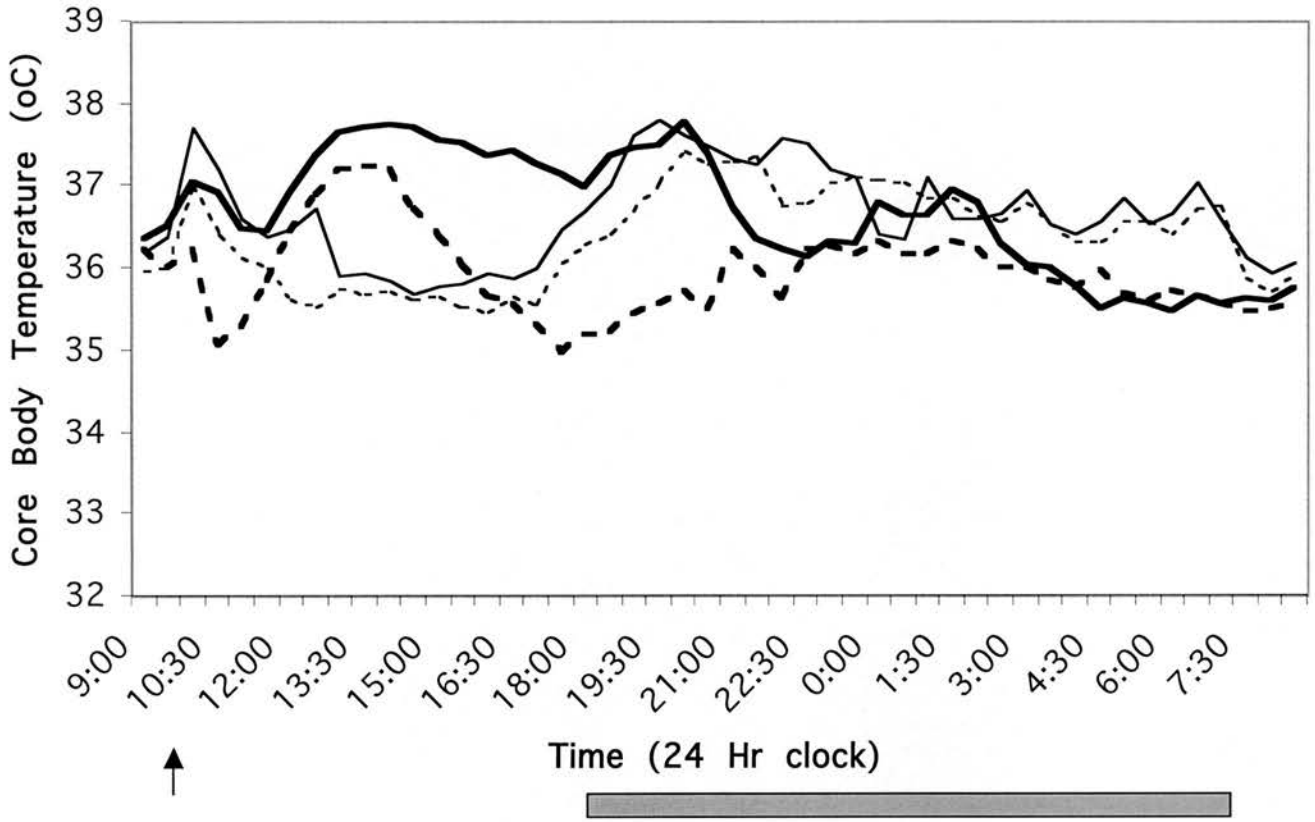

Figure 6.5 Core body temperature in transgenic and wild type mice treated with a single i.p dose of 30mg.kg^{-1} MDMA
Data is expressed as pooled data for each 30minute period after injection and the means of 6-8 animals .
Groups: ——— =nontransgenic saline, ——— =nontransgenic MDMA , - - - - =transgenic saline - - - - - =transgenic MDMA
The injection was given intraperitoneal (arrow) at 10am .The graph covers the 24 hr period9am-9am , the darkperiod is indicated by the grey bar

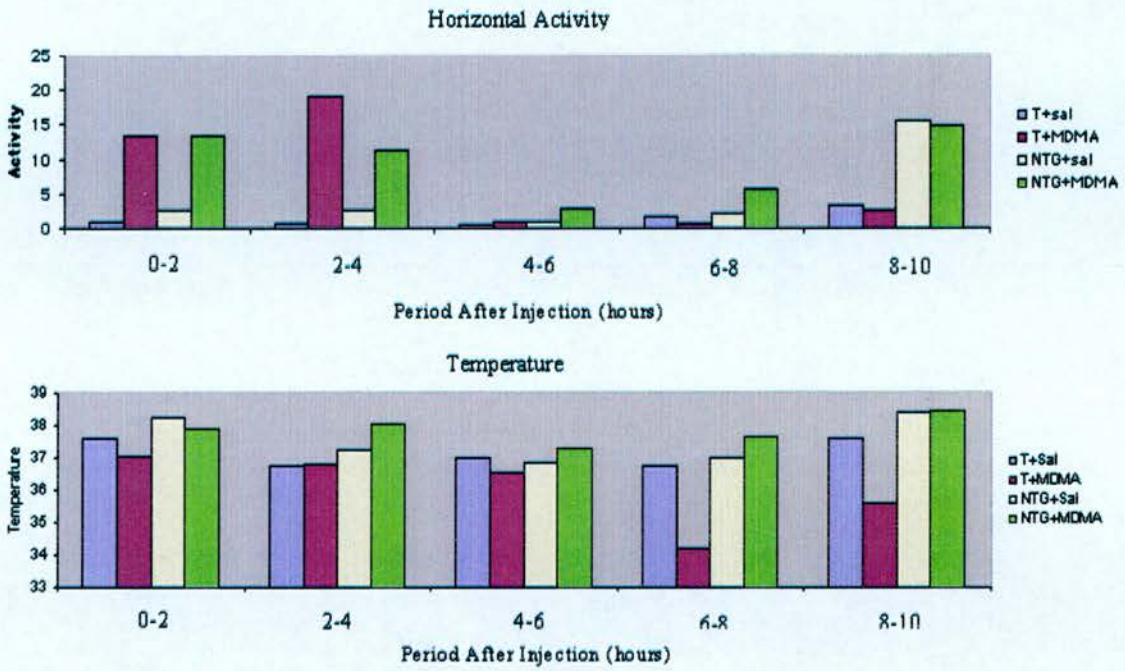

Figure 6.6 Effect of a single dose of 10mg.kg^{-1} MDMA on core body temperature and horizontal activity in transgenic and wildtype mice.

Data is expressed as pooled data for each two hour period after injection and the means of 6-8 animals . T= transgenic, NTG=wildtype

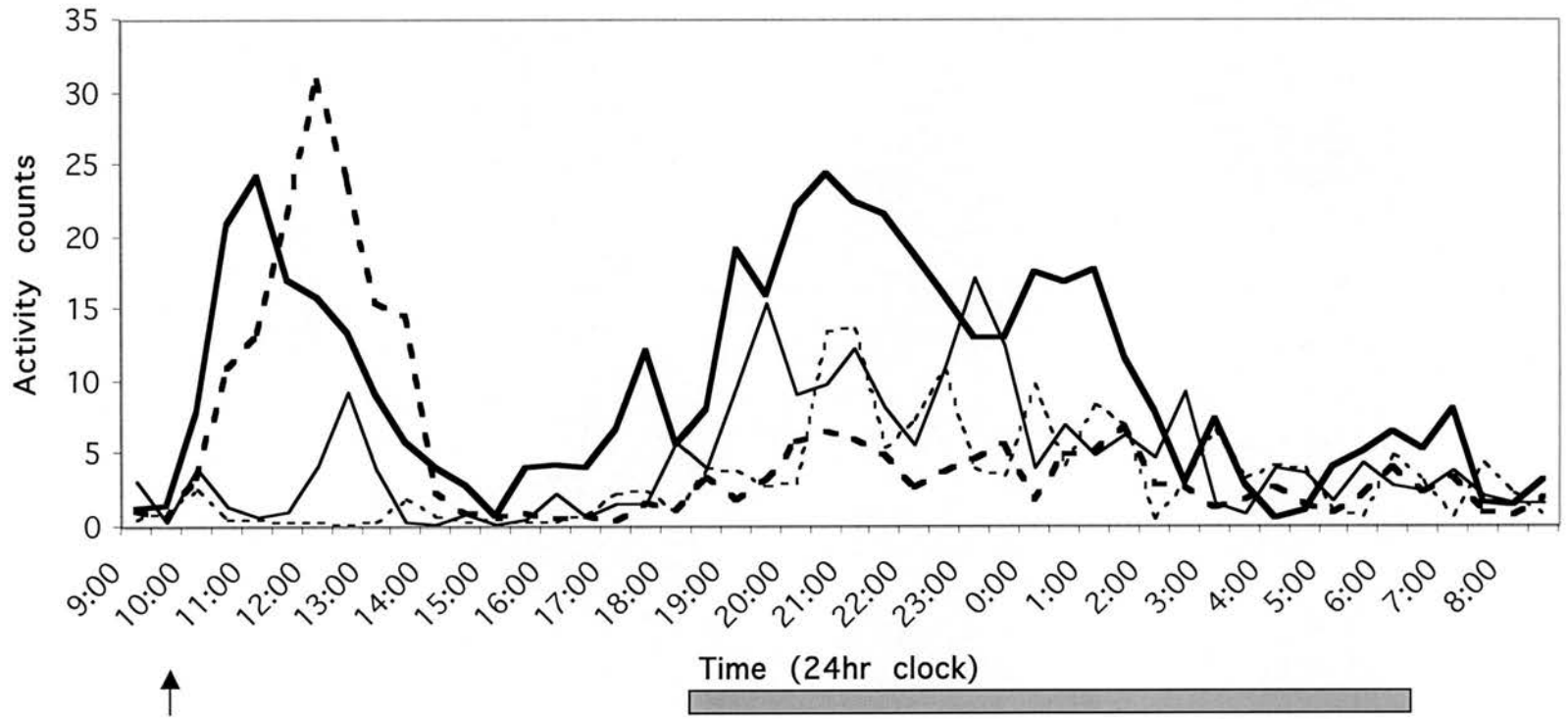

Figure 6.7 Time course of the effect of a single dose of 10mg.kg-1 MDMA on horizontal activity in transgenic and wild type mice. Data is expressed as the mean of 6-8 animals and as the mean of 30 minutes of measurements for each animal (measurements sampled every 10 minutes).The graph covers the 24 hr period 9am-9am , the dark period is indicated by the grey bar

Groups: ____=nontransgenic saline,—— =nontransgenic MDMA , - - - - =transgenic saline - - - - - =transgenic MDMA

the onset of circadian night, when activity returned to normal. The MDMA-induced increase was greater in transgenic than wild-type mice.

Immediately following injection of the higher dose of MDMA (30 $mg.kg^{-1}$), wild-type animals exhibited an increase in locomotor activity that lasted for 6 hours (Figure 6.8). There was a similar response of the same duration in transgenic animals, but unlike the response to the lower dose of MDMA, the response was less pronounced in transgenic animals than in wild-type animals (Figures 6.8 and 6.9).

The locomotor response peaks during the first three hours after injection. Two-way ANOVA was therefore used to compare the cumulative activity over the three hours following MDMA administration on all five treatment days; group, treatment and the interaction were all significant factors ($p< 0.001$). Figure 6.10 shows the locomotor response to MDMA across the five treatment days. The dose-response curve for MDMA in transgenic animals shows a shift to the left, with the peak locomotor response to MDMA occurring at 10 $mg.kg^{-1}$ in transgenic animals, but at 30 $mg.kg^{-1}$ in wild-type animals. Least square means followed by Student-Newman-Keuls comparison showed that drug-naïve transgenic animals were no less active than wild-type animals and that MDMA increased the activity in both groups ($p<0.05$). The locomotor response to MDMA at 10 $mg.kg^{-1}$ was significantly increased compared to saline-treated controls for both transgenic mice ($p<0.001$) and wild-type mice ($p=0.003$). At this dose there is no significant difference in the effect of MDMA between the two genotypes. At 30 $mg.kg^{-1}$ both wild-type ($p=0.001$) and transgenic ($p=0.026$) mice show significant increases in locomotor activity compared to saline-treated controls. There is also a significant difference ($p=0.04$) between transgenic and wild-type mice in the response to MDMA.

One further consideration in differences between the transgenic and wildtype mice at high doses of MDMA is the possibility of increased neurotoxicity in transgenic mice due to the presence of the human transporter. No neurodegenerative markers could be analysed in these experiments , however it is possible that loss of available 5-HT neurons after 10 $mg.kg^{-1}$ MDMA might inhibit or alter the apparent behavioural

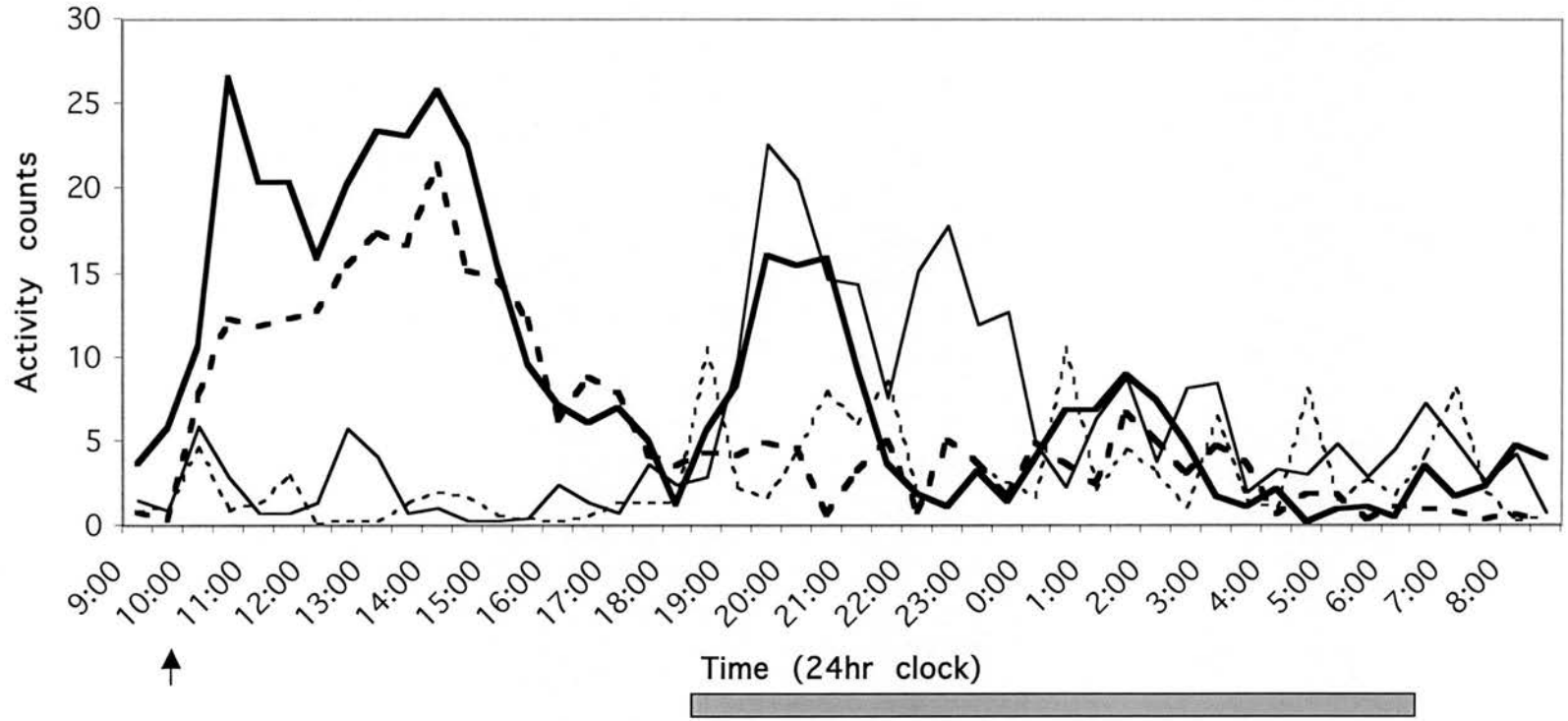

Figure 6.8 Time course of the effect of a single dose of 30mg.kg-[1] MDMA on horizontal activity in transgenic and wild type mice.
Data is expressed as the mean of 6-8 animals and as the mean of 30 minutes of measurements for each animal (measurements sampled every 10 minutes).The graph covers the 24 hr period9am-9am , the darkperiod is indicated by the grey bar
Groups: —— =nontransgenic saline, ___ =nontransgenic MDMA , - - - - =transgenic saline - - - - - =transgenic MDMA

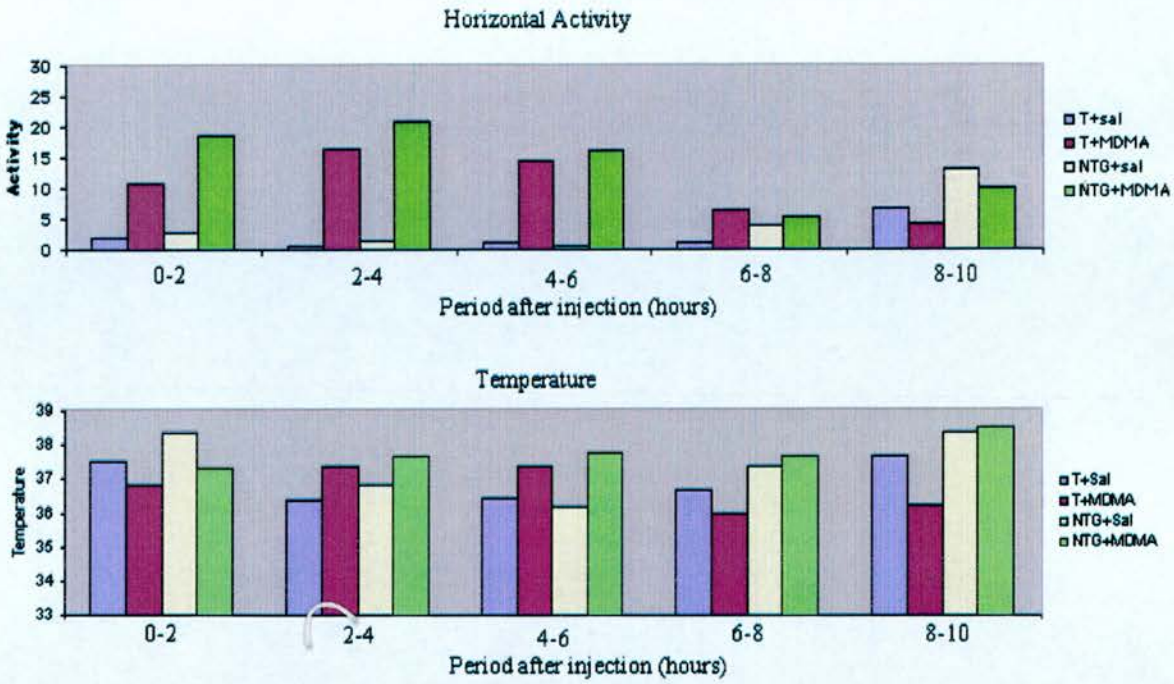

Figure 6.9 Affect of a single dose of 30mg.kg-1 MDMA on core body temperature and horizontal activity in transgenic and wildtype mice.

Data is expressed as pooled data for each two hour period after injection and the means of 6-8 animals . T= transgenic, NTG=wild type

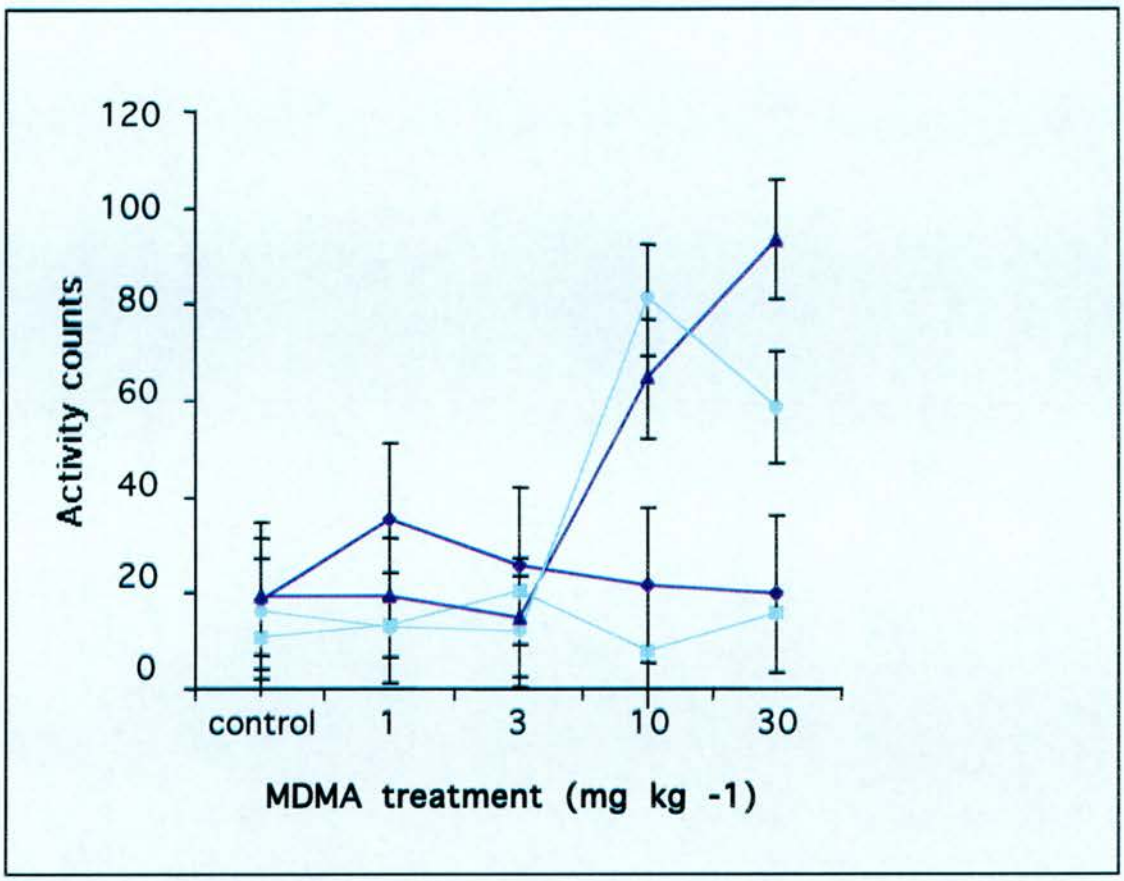

Figure 6.10 Mean locomotor activity in the 2hr period after injection of MDMA in wild-type and transgenic mice.

Treatment was by a single i.p. injection of MDMA or saline once daily. Increasing doses were given on subsequent days. Control = drug naïve mice

◆ = wild-type –saline ▲ = wild-type MDMA ■ =transgenic saline
● =transgenic MDMA

effects of the higher dose. Further analysis by HPLC or immunocytochemical analysis of the 5-HT pathways of transgenic mice after a single dose would further clarify this argument. However the quick return to baseline behaviour for transgenic mice after 72 hrs washout suggets that any degeneration iss minor.

6.3.5 Other behavioural observations during the experiment

Mice were weighed before the experiment and during the week of the injections in order to accurately dose the animals and to monitor their welfare. The mice were age-matched and compared to their littermates. Transgenic mice were consistently and significantly lighter than their non-transgenic littermates (wild-type = 31.2 ± 1.1 grams, transgenic = 26.9 ± 0.48 grams, $p<0.01$). Mice were observed at intervals during the hypothermic response to check on their welfare.

6.4 Discussion

The temperature results can be interpreted by assuming a biphasic effect of MDMA on body temperature, with doses of MDMA up to 10 $mg.kg^{-1}$ evoking a dose-dependent reduction in body temperature and higher doses reversing this effect (See Figure 6.11, a hypothetical model of temperature-dose response for MDMA in mice.) This interpretation would explain why many of the transgenic animals responded maximally to 10mg. kg^{-1} MDMA and with a smaller hypothermic response to higher doses of the drug. Among those animals that displayed a hypothermic response only at the highest dose of MDMA, some may have variations in brain 5-HT concentrations that led them to respond to MDMA only at higher doses.

6.4.1 MDMA effects in wild-type mice

MDMA caused temperature and locomotor effects in wild-type mice that were dose dependent. The time course and magnitude of these responses corresponded with those reported previously in mice after repeated doses of MDMA

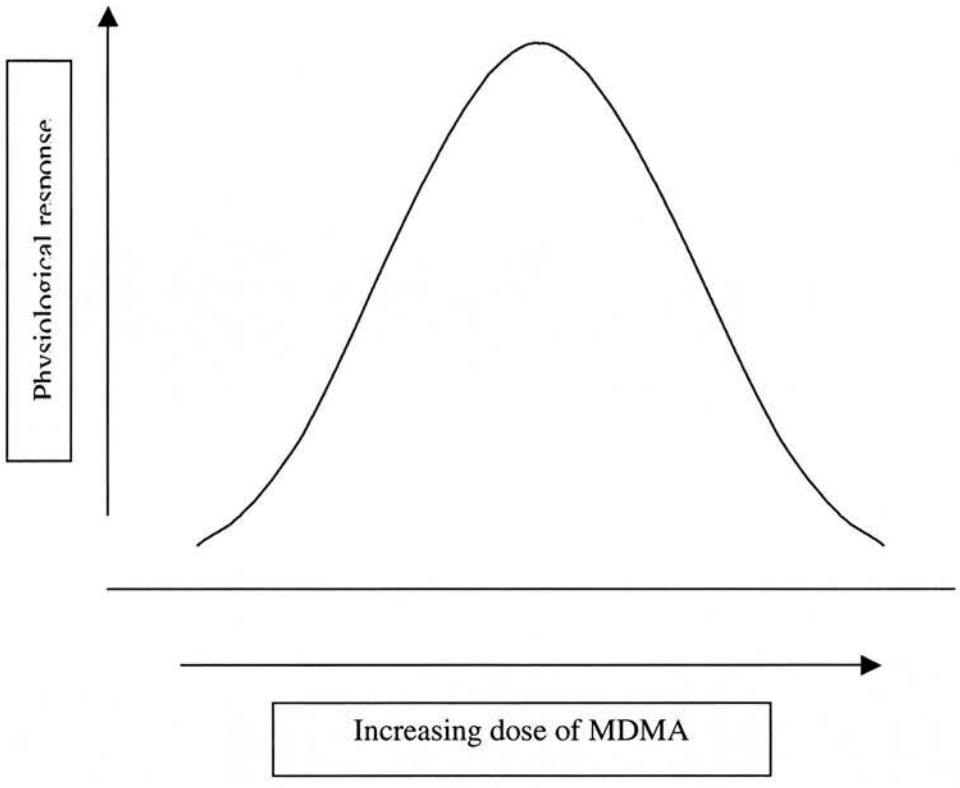

Figure 6.11 Model of MDMA induced dose-response in mice

(Miller and O'Callaghan, 1995; O'Callaghan J, 1994; O'Shea E, 2001) or where an increase in horizontal locomotor activity and a slight hypothermia occurred, followed by hyperthermia (Miller D, 1994; Miller and O'Callaghan, 1995; O'Callaghan J, 1994; O'Shea E, 2001).

6.4.2 MDMA and locomotor activity in transgenic mice

In drug-naïve transgenic mice there was a distinct reduction in the level of horizontal locomotor activity during the dark period of the diurnal cycle. The increase in locomotor activity in response to MDMA was similar to that in wild-type mice but peaked at a lower dose of MDMA. In contrast, in 5-HTT knockout mice, the locomotor affect is abolished, though there are no reports of the effects of MDMA on body temperature in these animals (Bengel, 1998). This reports confirms that the locomotor effects of MDMA are dependent upon the presence of the 5-HTT and probably on carrier-mediated 5-HT release rather than absolute 5-HT levels, which are reduced in both transgenic and knockout animals.

Transgenic mice had a tendency to be smaller than their non-transgenic littermates, despite their reduced locomotor activity. Reduced body weight is a common non-specific characteristic of many transgenic mice, but in combination with reduced activity levels lower body weight might indicate an alteration caloric intake by these 5-HTT over-expressing mice. The possible role of hypothalamic 5-HT in appetite (Hernandez et al., 1991) and eating disorders (Kaye et al., 1998), and the controversial use of the 5-HT-releasing amphetamine fenfluramine as an appetite suppressant (Curzon and Gibson, 1999; Garattini et al., 1986), suggest that these mice may provide an animal model for studies on eating disorders.

6.4.3 MDMA and thermoregulation in transgenic mice

In wild-type mice, treatment with moderate doses of MDMA causes 5-HT release and increased extracellular 5-HT, which normally causes a hyperthermic response mediated via postsynaptic 5-HT_2 receptors. However, very high MDMA doses cause

a massive 5-HT release, which may have the same effect as injections of high doses of 5-HT to the hypothalamus, thus stimulating 5-HT_{1A} autoreceptors and overriding 5-HT_2-induced hyperthermia (de Roij et al., 1979).

In transgenic mice with over-expression of the 5-HTT there are more sites for carrier-mediated 5-HT release, thus increasing the likelihood of 5-HT release at lower concentrations of MDMA. Transgenic mice have a slightly altered affinity for MDMA as their temperature response peaks at 10 $mg.kg^{-1}$ but is still increasing at 30 $mg.kg^{-1}$ in wild-type animals. Neither wild-type nor transgenic mice responded to the next lower dose of MDMA (3 $mg.kg^{-1}$).

The pattern of the hypothermic response in transgenic mice could result from one or more of a number of factors. Firstly, 5-HT receptor balance could be altered as a result of either greater 5-HT release or adaptive changes in 5-HT receptor expression or sensitivity during development. Secondly, 5-HT could be depleted because of greater carrier-mediated release. Thirdly, 5-HT could interact with, or change, other transmitters, such as DA and NA. Fourthly, peripheral responses could be altered, either directly in the heat regulatory system or indirectly through corticosterone production. Finally, one parameter may be behavioural changes in the response of the animal to heat-challenge, as seen with the hyperthermia induced by MDMA.

MDMA induces locomotor activity; increased activity may lead to heat loss through vasodilation, causing the animals to curl up and stay still to preserve heat though no observations were made during this time period. At the higher dose of MDMA, it is possible that the cold effect is reduced (locomotor activity is reduced) or the signal received more quickly and so the heat preservation behaviour occurs, preventing prolonged hypothermia. This alteration may be caused by any of the above factors, particularly alterations in peripheral thermoregulation and vasodilation. 5-HT is vasoconstrictive in large vesssels via 5-HT_2 receptors, but also vasodilatory through other non-5-HT_2 receptors (Van Nueten et al., 1984). 5-HT_2 receptor stimulation in the periphery can also cause hypothermia in mice. Here, both possible changes in peripheral 5-HT receptors and altered central feedback could be involved. The

observation that transgenic mice with hypothermia showed piloerection and symptoms of high blood pressure (e.g., raised intraocular pressure) would suggest that peripheral changes are occurring. It would be of interest here to study the response to a heat challenge in transgenic mice. Part of the hypothermic response may not be a direct effect of 5-HT or dopamine release on 5-HT or dopamine receptors, but an altered sensitivity to heat. MDMA has been shown to alter thermoregulatory responses to heat in rats (Dafters RI, 1998). These mice may be responding to the thermogenic action of MDMA with a compensatory hypothermia.

Central nervous system control of thermoregulation may also be involved in the response to MDMA. In the brain of transgenic mice, the balance of 5-HT_{1A} and 5-HT_{2A} receptor activation may be perturbed by increased carrier-mediated release of 5-HT through the 5-HTT. This perturbation may be sufficient to stimulate 5-HT_{1A} receptors to induce hypothermia and override the 5-HT_{2A}-mediated hyperthermia. In addition, the balance of expression of the two receptors may be altered by the transgene such that the reduction in 5-HT levels in these animals sensitises 5-HT_{1A} receptors or desensitises 5-HT_2 receptors. In 5-HTT knockout mice the desensitisation of 5-HT_{1A} receptors in the raphé is different from that in the hippocampus, despite a drastic decrease in 5-HT in all areas (Li et al., 2000; Li et al., 1999a). These findings indicate that it would be possible for the 5-HT_{1A} receptors in transgenic mice to also be differentially affected. The exact cause of 5-HT_{1A} receptor desensitisation in 5-HTT knockout mice has not been determined but may result from the decline in 5-HT or a direct effect of the 5-HTT on the expression. However, it is plausible to predict that the increase in 5-HTT has caused a commensurate increase in 5-HT_{1A} levels because of increased transport and so reduced feedback. There is some discussion as to whether 5-HT_{1A} presynaptic receptors are active under baseline conditions (see Introduction) If not, 5-HT_{1A} receptors may have a greater capacity to respond to change as a feedback system than postsynaptic receptors.

In addition to increases in 5-HT_{1A} sensitivity or expression, the postsynaptic 5-HT_2 receptors may be desensitised. As discussed in the previous chapter, an over-

expression of 5-HTT may lead to greater release at the level of the synaptic cleft, which in turn may desensitise postsynaptic receptors. Either or both of these receptor changes would predispose 5-HTT transgenic mice to hypothermia in response to 5-HT release. This effect would be more pronounced when combined with augmented MDMA-induced 5-HT release

The higher dose (30 $mg.kg^{-1}$) of MDMA did not cause such prolonged or severe hypothermia in those transgenic mice that responded at the lower dose (10 $mg.kg^{-1}$). This may attributed either to desensitisation of the $5\text{-}HT_{1A}$ receptors or to a depletion of available 5-HT. High doses of MDMA may cause depletion of 5-HT stores, as these mice probably have greater release than normal at the 10 $mg.kg^{-1}$ dose, but there is possibly not as much 5-HT available for release at the higher dose of MDMA. It is also possible that stored 5-HT levels have not recovered after the MDMA-stimulated release, but the 6- to 24-hour period before recovery of 5-HT levels in normal mice after 5-HT release (Logan, 1988; Stone, 1987) gives only qualified support to this hypothesis. Here it is possible that the presence of the human transporter may be a contributing factor; in primates 5-HT recovery is slower than in mice (Slikker et al., 1989).

In addition to exerting direct effects on the 5-HT system, MDMA stimulates central DA release via 5-HT stimulation of $5\text{-}HT_{1B}$ receptors in mice; this response is attenuated in both 5-HTT (Bengel, 1998) and $5\text{-}HT_{1B}$ knockout mice (Scearce-Levie, 1999). $5\text{-}HT_{2A}$ and $5\text{-}HT_{2B}$ receptors tonically inhibit dopamine release in rats (Di Matteo et al., 1998; Millan et al., 1998), which may be altered in transgenic mice and so alter metabolic rate and locomotor activity. These changes in metabolic rate and locomotor activity may be sufficient to counteract a centrally induced hypothermia. The increased locomotor activity after injection with MDMA may also explain the late onset of the hypothermia, which coincides with the activity returning to baseline.

In summary, these results show that over-expression of the 5-HTT reduces baseline locomotor activity in mice during the circadian night and disturbs the thermoregulatory response to MDMA. Transgenic mice have a profound

hypothermic response to MDMA, in contrast to the very slight hyperthermia seen in wild-type animals. Transgenic mice have an increased sensitivity to MDMA, with a peak locomotor response at lower dose than wild-type mice. There are several possible mechanisms for the hypothermic and locomotor responses.

Chapter 7

Summary and Conclusions

This thesis has described the production of transgenic mice over-expressing the human 5-HTT. These mice express 2-3 times the total 5-HTT protein of wild-type mice, in a pattern similar to that of the endogenous protein in mice and humans. This over-expression leads to a reduction in total 5-HT levels of the brain, as well as altered behavioural and pharmacological responses to MDMA.

7.1 5-HT knockout mice

Studies of 5-HTT knockout mice have provided a wealth of information about the functions and regulation of the 5-HT system. These mice have no 5-HTT expression and hence no specific 5-HT uptake. The removal of the 5-HTT is not fatal but has several effects on the 5-HT system. Knockout mice had vesicular 5-HT stores reduced by about 95% (Bengel, 1998) because of a loss of released transmitter. The knockout mouse had extracellular 5-HT levels about six-fold higher than those seen in wild type littermates, similar to those seen in wild type animals after citalopram treatment (Bengel, 1998; Fabre et al., 2000). Concentrations of 5-HIAA were slightly decreased in the knockout mice in most brain areas tested, but the decrease in 5-HIAA was not of the same magnitude as the decrease in tissue 5-HT, so that the 5-HIAA/5-HT ratio was 130-180% of wild type. The 5-HTT heterozygous mice expressed the 5-HTT at levels 50% of those in wild type animals, but did not have altered 5-HT levels, suggesting that feedback systems are able to compensate for a decrease in 5-HTT expression.

The knockout of the 5-HTT altered the expression of several 5-HT receptor subtypes (5-HT_{1A} 5-HT_{1B} and 5-HT_2) in the brain, although it is not known whether this resulted from the effects of altered 5-HT concentrations, or from developmental compensations. Many of the 5-HT receptors are expressed during development; 5-HTT itself is expressed well before the formation of neurons and in non 5-HT producing cells, where the transporter probably takes up 5-HT as a mitogenic factor.

The alterations in 5-HT receptor expression seen in 5-HTT knockout mice are brain region- and gender-specific. In the case of the 5-HT_{1A} receptor, several studies (Fabre et al., 2000; Li et al., 2000; Li et al., 1999a) have shown that the receptor density and mRNA is reduced in the dorsal raphé and that this reduction is greater in knockout than in heterozygous mice. In contrast, the hippocampus shows a slight increase in 5-HT_{1A} expression of equal magnitude in both knockout and heterozygote, whilst other brain areas show no changes. The behavioural and neuroendocrine effects of 5-HT_{1A} agonists are also significantly reduced in knockout mice (Li et al., 2000; Li et al., 1999a) but not in heterozygotes. Electrophysiological studies of the 5-HT system showed that 5-HTT deletion increased the recovery time of the firing rate of hippocampal CA(3) pyramidal neurons following iontophoretic applications of 5-HT (Gobbi et al., 2001). Whilst both 5-HTT knockout mice and heterozygous mice have desensitised presynaptic 5-HT_{1A} receptors, only the knockout mice display any post synaptic 5-HT_{1A} receptor desensitisation (Gobbi et al., 2001; la Cour et al., 2001). The 5-HT_{1B} autoreceptor is also reduced in the knockout mouse in the substantia nigra but not other brain regions. 5-HT_{2A} receptors are also altered in a brain region- specific manner, with decreases in the cerebral cortex and lateral striatum and a new zone of expression in the outer striatum.

In addition to changes in the expression of 5-HT receptors, the 5-HTT knockout mouse exhibits altered behavioural and pharmacological responses to drug challenge, with a reduced locomotor response to MDMA. (Bengel, 1998) and an increased cocaine place preference (Hall et al., 2002; Sora et al., 1998). The locomotor effects of MDMA are also abolished in 5-HT_{1B} receptor knockout mice, suggesting that this effect in transporter knockout mice may be due to receptor desensitization as much as to direct effects on 5-HT in the synapse.

The regional specificity of the changes in receptor expression and the fact that the heterozygote, although showing a 50% reduction in transporter, does not have reduced tissue 5-HT concentrations or an altered neuroendocrine response to 5-HT_{1A} receptor agonists, suggests the involvement of a complex loop in the regulation of the 5-HT system.

7.2 The 5-HTT over-expressing mice

The 5-HTT knockout mouse shows reduced tissue 5-HT concentrations, increased extracellular 5-HT, desensitised 5-HT_{1A} and 5-HT_2 receptors and reduced locomotor response to MDMA. It might be expected that increased 5-HTT expression in the transgenic mice would lead to changes opposite to those seen in 5-HTT knockout mice: increased intracellular 5-HT and decreased extracellular 5-HT, coupled with increased 5-HT_{1A} receptor sensitivity and increased 5-HT_{2A} and 5-HT_{1B} receptor sensitivity. As the changes are brain region specific it would be expected that the 5-HTT transgenic mice would show the greatest variations in 5-HT levels in the DRN and hippocampus.

The expected increase in 5-HT did not occur in the over-expressing mice; indeed, they showed a reduction in total brain 5-HT. This reduction is not completely unexpected for several reasons. The total ablation of the 5-HTT in knockout mice is a major perturbation compared to the 2-3-fold increase in 5-HTT expression seen in transgenic mice. The decrease in brain 5-HT concentrations associated with a small increase in transporter protein would suggest that, whilst the expression of the 5-HTT is required for 5-HT uptake and storage, a minor disturbance in 5-HTT expression has complex effect on 5-HT homeostasis. Such a complex effect is probably mediated through several interlocking pathways. One hypothesis is that an acute increase in the 5-HTT leads to reduced 5-HT in the synapse. At the soma extracellular 5-HT is probably also reduced by increased 5-HTT activity. As a consequence, the presynaptic autoreceptors at both the synapse and soma receive less stimulation, leading to increased 5-HT release upon nerve stimulation. This feedback mechanism, which compensates for the reduced 5-HT due to increased transporter activity, is present in transgenic mice from conception. This hypothesis agrees to some extent with pharmacological data. Chronic SSRI treatments to block the 5-HTT have very different effects on the 5-HT system, including neuronal firing, than acute treatment (Benmansour et al., 2002). Evidence shows that chronic SSRI treatments achieve much of their therapeutic effect after a lag period of 21 days, due

to down regulation of the 5-HTT rather than to acute increases in synaptic 5-HT levels(Benmansour et al., 2002). Another factor contributing to reduced 5-HT levels as in the knockout mouse may be direct effects of the increased 5-HTT on autoreceptors, possibly due to changes in developmental expression.

One of the aims of this type of research is to provide a model for studying the effects of a single factor on possible behavioural and psychiatric states in humans. By comparing the genetic, neurochemical and behavioural effects of various agents in animal models with the known psychiatric or disease states in humans, researchers can extrapolate from animal data which areas of genetic or neurochemical disturbance might be involved in human disease. The findings of this thesis research show that increased 5-HTT expressions have far-reaching effects on pharmacological and behavioural responses in transgenic animals. The effect of MDMA on locomotor function and temperature regulation in transgenic mice may be direct (through the 5-HTT) or indirect (via variations in receptor sensitivity or dopamine release). These effects in transgenic mice are different to those in wild-type mice. This difference alone does not mean that the baseline state in transgenic mice is altered compared to wild-type mice, just that the two strains respond differently to challenges. The reduced circadian locomotor activity in transgenic mice in the absence of MDMA treatment is better evidence that 5-HTT over-expression is sufficient to alter behaviour and possibly therefore disease.

Although there are few other models of over-expression in the 5-HT system, mice over-expressing the 5-HT_3 receptor have been produced and studied (Allan et al., 2001). This receptor is thought to be involved in the reward pathway, by controlling DA release in the mesolimbic system. Transgenic mice over-expressing the 5-HT_3 receptor were made in order to study the receptor's role in addiction. Like their 5-HTT over-expressing counterparts, the 5-HT_3 over-expressing mice had a relatively small increase in receptor expression, which produced altered pharmacological responses. Their sensitivity to ethanol was enhanced (Engel and Allan, 1999; Engel et al., 1998) and their place preference for cocaine was reduced, whilst their sensitivity to cocaine and DA release in response to cocaine were

increased (Allan et al., 2001). These findings are further evidence that significant behavioural effects can result from changing gene expression only 2- to 3-fold. Such relatively small changes, which could occur as a consequence of normal genetic variation, are sufficient to cause neurochemical changes that could result in disease.

The monoamine hypothesis of depression and anxiety posits that affective disorders are due to reduced activity of the 5-HT system, which could result from reduced availability of 5-HT, or reduced responsiveness to the neurotransmitter, or its increased removal. Evidence suggests that in some subsets of depressed patients the 5-HTT is increased (Dahlstrom et al., 2000), but considerable evidence from other studies suggests that the 5-HTT is reduced (Malison et al., 1998; Staley et al., 1998). These conflicting results can be explained by the different criteria for depression used in these studies, as described in the Introduction (see Section 1.6.1).

The findings from this thesis research confirm that the role of the 5-HTT in the 5-HT system is more complex than taking up extracellular 5-HT, thus terminating the 5-HT neurotransmission. In addition to the direct re-uptake from the synapse, the transporter indirectly affects other elements of the 5-HT neuron, such as the receptor changes suggested above. The development of the 5-HT system was not hugely disturbed in the 5-HTT transgenic mouse, but a small increase in 5-HTT expression was sufficient to cause measurable behavioural effects. Such disturbances could explain why humans with slight variations in 5-HTT expression might be prone to developing affective disorders. The transgenic mouse model used here provides some insights into possible areas of interaction within the 5-HT system. For instance, studies on the localisation and activity on both pre- and postsynaptic receptors would provide information about the effects of changes in transporter expression on receptor function. This information might be useful in understanding and improving treatment of affective disorders, in which the 5-HT_{1A} receptor agonist pindolol has been implicated in enhancing SSRI treatment (Pineyro et al., 1994). Studies with the 5-HTT over-expressing animal model might confirm this interaction. Postsynaptic receptor function could also be examined in this model by looking, for instance, at the localisation and sensitivity of the 5-HT_2 receptors and at

their downstream effects, such as temperature regulation or neuronal activity (Morishima and Shibano, 1995; Nash et al., 1989; Sugimoto et al., 1991).

7.3 Future work

In this research the synaptic concentration of 5-HT in the over-expressing mice was not determined. The use of micro-dialysis to analyse these levels would provide more information as to which mechanisms are involved in reducing 5-HT levels. Another factor contributing to reduced 5-HT levels may be direct effects of the increased 5-HTT on autoreceptors, possibly due to changes in developmental expression.

Further work on 5-HT receptor availability, receptor sensitivity, and synaptic concentrations of 5-HT in these over-expressing mice would clarify the factors involved in regulating the availability of 5-HT. Postsynaptic 5-HT receptors would also be affected by altered synaptic levels of 5-HT, thus localization and expression of 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2A} receptors at both the protein and RNA level would be of the greatest importance in the understanding of the regulation of the 5-HT system.

One further useful study would be the autoradiographic localisation and quantification of the 5-HTT in transgenic mice. Such a study is important for several reasons. Firstly, the pharmacological binding data (Chapter 4) suggest that the increases seen in the cortex are greater than in the midbrain, and therefore the soma, which may be an important consideration in interpreting any behavioral and systemic results. Secondly, the 5-HTT has been shown to be present in glia (Zhou et al., 1998), and it is possible that some of the increased cortical expression seen in the transgenic mice is partly in these cells rather than the neurons. A third consideration would be the distribution of 5-HTT expression within individual neurons. Recent electron microscopy studies (Zhou et al., 1998) suggest that the 5-HTT is found perisynaptically, and increasing its concentration might alter its micro-localisation.

A final matter of interest is the study of the developmental changes in early gene expression which are altered in transgenic mice. This would be helpful in elucidating both the role of the 5-HTT in brain development and the effects of over-expression on the 5-HT system.

7.4 Use of transgenic mice as a tool for understanding the complex interactions of the 5-HT system.

The over-expression of 5-HTT in transgenic mice offers a better animal model of depression in man than the 5-HTT knockout mouse, though it cannot be considered an appropriate model of all types of affective disorder. Transgenic mice over-expressing the 5-HTT also provide a good model for further studies on interactions of various parts of the 5-HT system, particularly those aspects that respond to SSRI treatments. One example would be 5-HT_{1A} receptor sensitivity (Pineyro et al., 1994), where the effects of a lifetime change would probably differ greatly from those of an acute pharmacological model.

5-HTT transgenic mice could also be useful for studying the interactions between the 5-HT system and other neurotransmitter systems, such as the dopaminergic system, particularly in areas such as the striatum where 5-HT_{1B} receptors are known to influence DA neuronal firing (Sarhan et al., 2000). The evidence for locomotor disturbance in the transgenic mice (see Section 6.1.2) and loss of locomotor stimulation in response to MDMA in knockout mice (Bengel, 1998) suggests that these or other dopaminergic neurons might also altered by variations in 5-HTT expression. The transgenic model provides a good system to study these types of interactions. Comparative studies on the localisation of the 5-HT projections areas of the adult forebrain could be done with transgenic and wild type mice. If differences are found, then localisation of 5-HT pathways could be studied throughout development. Such localisation studies would enhance the understanding not only of affective disorders, but also disturbances in serotonergic metabolism such as anorexia nervosa. This eating disorder is a possible area of study as these mice

appear to be smaller than their wild-type littermates, and recent evidence in 5-HTT knockout mice has shown gastric disturbance (Chen et al., 2001).

7.5 Conclusion

In conclusion, this thesis research has produced a transgenic mouse model in which the moderate over-expression of one gene has had important ramifications for the whole animal, both at the neurochemical and behavioural levels. The RNA and protein expression studies confirm the validity of this transgenic method as a reliable model for human protein expression. The two main findings from this research are that the over-expression of the 5-HTT leads to reduced total brain 5-HT stores and to reduced locomotor activity and disturbed thermoregulation in response to MDMA. These results have stimulated the generation of some hypotheses for how 5-HTT over-expression might affect the 5-HT system as a whole, with implications for possible roles of the 5-HTT in human affective disorders. This genetic manipulation of the 5-HTT has produced an animal model that will be a valuable tool for further studies in the 5-HT system.

References

Anonymous (1973). Fenfluramine--another appetite suppressant. Med Lett Drugs Ther *15*, 33-34.

Adell, A., and Artigas, F. (1998). A microdialysis study of the in vivo release of 5-HT in the median raphe nucleus of the rat. Br J Pharmacol *125*, 1361-1367.

Aghajanian, G. K. (1972). Chemical-feedback regulation of serotonin-containing neurons in brain. Ann N Y Acad Sci *193*, 86-94.

Aghajanian, G. K., and Lakoski, J. M. (1984). Hyperpolarization of serotonergic neurons by serotonin and LSD: studies in brain slices showing increased K+ conductance. Brain Res *305*, 181-185.

Albert, P. R., Lembo, P., Storring, J. M., Charest, A., and Saucier, C. (1996). The 5-HT1A receptor: signaling, desensitization, and gene transcription. Neuropsychopharmacology *14*, 19-25.

Allan, A. M., Galindo, R., Chynoweth, J., Engel, S. R., and Savage, D. D. (2001). Conditioned place preference for cocaine is attenuated in mice over-expressing the 5-HT(3) receptor. Psychopharmacology (Berl) *158*, 18-27.

Anderson, G. M., and Horne, W. C. (1992). Activators of protein kinase C decrease serotonin transport in human platelets. Biochim Biophys Acta *1137*, 331-337.

Arancibia, S., Rage, F., Astier, H., and Tapia-Arancibia, L. (1996). Neuroendocrine and autonomous mechanisms underlying thermoregulation in cold environment. Neuroendocrinology *64*, 257-267.

Araneda, R., and Andrade, R. (1991). 5-Hydroxytryptamine2 and 5-Hydroxytryptamine 1A receptors mediate opposing responses on membrane excitability in rat association cortex. Neuroscience *40*, 399-412.

Asberg, M., Eriksson, B., Martensson, B., Traskman-Bendz, L., and Wagner, A. (1986). Therapeutic effects of serotonin uptake inhibitors in depression. J Clin Psychiatry *47 Suppl*, 23-35.

Ase, A. R., Reader, T. A., Hen, R., Riad, M., and Descarries, L. (2000). Altered serotonin and dopamine metabolism in the CNS of serotonin 5- HT(1A) or 5-HT(1B) receptor knockout mice. J Neurochem *75*, 2415-2426.

Azmitia, E., and Gannon, P. (1986). The Primate Serotonergic System: A Review of Human and Animal studies and a report on Macaca fascicularis. Advances in neurobiology *43*, 407-468.

Azmitia, E. C., Buchan, A. M., and Williams, J. H. (1978). Structural and functional restoration by collateral sprouting of hippocampal 5-HT axons. Nature *274*, 374-376.

Barden, N., Reul, J. M., and Holsboer, F. (1995). Do antidepressants stabilize mood through actions on the hypothalamic- pituitary-adrenocortical system? Trends Neurosci *18*, 6-11.

Barker, E. L., and Blakely, R. D. (1998). Structural determinants of neurotransmitter transport using cross- species chimeras: studies on serotonin transporter. Methods Enzymol *296*, 475-498.

Barker, E. L., Kimmel, H. L., and Blakely, R. D. (1994). Chimeric human and rat serotonin transporters reveal domains involved in recognition of transporter ligands. Mol Pharmacol *46*, 799-807.

Barnes, N. M., and Sharp, T. (1999). A review of central 5-HT receptors and their function. Neuropharmacology *38*, 1083-1152.

Barton, C. L., and Hutson, P. H. (1999). Inhibition of hippocampal 5-HT synthesis by fluoxetine and paroxetine: evidence for the involvement of both 5-HT1A and 5-HT1B/D autoreceptors. Synapse *31*, 13-19.

Battersby, S., Ogilvie, A. D., Smith, C. A., Blackwood, D. H., Muir, W. J., Quinn, J. P., Fink, G., Goodwin, G. M., and Harmar, A. J. (1996). Structure of a variable number tandem repeat of the serotonin transporter gene and association with affective disorder. Psychiatr Genet *6*, 177-181.

Bengel, D., Johren, O., Andrews, A. M., Heils, A., Mossner, R., Sanvitto, G. L., Saavedra, J. M., Lesch, K. P., and Murphy, D. L. (1997). Cellular localization and expression of the serotonin transporter in mouse brain. Brain Res *778*, 338-345.

Bengel, D., Murphy, D., Andrews, A.,Wichems, C.,Feltner, D., Heils, A., Mossner,R., Westphal, H., Lesch, K. P. (1998). Altered brain serotonin homeostasis and locomotor insensitivity to 3,4-Methylenedioxymethamphetamine ("ecstasy") in Serotonin Transporter- Deficient mice. Molecuar Pharmacology *53*, 649-655.

Benmansour, S., Cecchi, M., Morilak, D. A., Gerhardt, G. A., Javors, M. A., Gould, G. G., and Frazer, A. (1999). Effects of chronic antidepressant treatments on serotonin transporter function, density, and mRNA level. J Neurosci *19*, 10494-10501.

Benmansour, S., Owens, W. A., Cecchi, M., Morilak, D. A., and Frazer, A. (2002). Serotonin clearance in vivo is altered to a greater extent by antidepressant-induced downregulation of the serotonin transporter than by acute blockade of this transporter. J Neurosci *22*, 6766-6772.

Blakely, R. D., Berson, H. E., Fremeau, R. T., Jr., Caron, M. G., Peek, M. M., Prince, H. K., and Bradley, C. C. (1991). Cloning and expression of a functional serotonin transporter from rat brain. Nature *354*, 66-70.

Blakely, R. D., De Felice, L. J., and Hartzell, H. C. (1994). Molecular physiology of norepinephrine and serotonin transporters. J Exp Biol *196*, 263-281.

Blakely, R. D., Moore, K. R., and Qian, Y. (1993). Tails of serotonin and norepinephrine transporters: deletions and chimeras retain function. Soc Gen Physiol Ser *48*, 283-300.

Blakely, R. D., Ramamoorthy, S., Schroeter, S., Qian, Y., Apparsundaram, S., Galli, A., and DeFelice, L. J. (1998). Regulated phosphorylation and trafficking of antidepressant-sensitive serotonin transporter proteins. Biol Psychiatry *44*, 169-178.

Blasberg, R., and Lajtha, A. (1966). Heterogeneity of the mediated transport systems of amino acid uptake in brain. Brain Res *1*, 86-104.

Blier, P., and Bergeron, R. (1998). The use of pindolol to potentiate antidepressant medication. J Clin Psychiatry *59*, 16-23; discussion 24-15.

Blier, P., Pineyro, G., el Mansari, M., Bergeron, R., and de Montigny, C. (1998). Role of somatodendritic 5-HT autoreceptors in modulating 5-HT neurotransmission. Ann N Y Acad Sci *861*, 204-216.

Bligh, J., and Hensel, H. (1974). Modern theories on the location and function of the thermoregulatory centres in mammals including man. Prog Biometeorol *1*, 413-433, 683-417.

Bligh-Glover, W., Kolli, T. N., Shapiro-Kulnane, L., Dilley, G. E., Friedman, L., Balraj, E., Rajkowska, G., and Stockmeier, C. A. (2000). The serotonin transporter in the midbrain of suicide victims with major depression. Biol Psychiatry *47*, 1015-1024.

Boulay, D., Depoortere, R., Perrault, G., Borrelli, E., and Sanger, D. J. (1999a). Dopamine D2 receptor knock-out mice are insensitive to the hypolocomotor and hypothermic effects of dopamine D2/D3 receptor agonists. Neuropharmacology *38*, 1389-1396.

Boulay, D., Depoortere, R., Rostene, W., Perrault, G., and Sanger, D. J. (1999b). Dopamine D3 receptor agonists produce similar decreases in body temperature and locomotor activity in D3 knock-out and wild-type mice. Neuropharmacology *38*, 555-565.

Bowker, R. M., Westlund, K. N., and Coulter, J. D. (1982a). Origins of serotonergic projections to the lumbar spinal cord in the monkey using a combined retrograde transport and immunocytochemical technique. Brain Res Bull *9*, 271-278.

Bowker, R. M., Westlund, K. N., Sullivan, M. C., and Coulter, J. D. (1982b). A combined retrograde transport and immunocytochemical staining method for demonstrating the origins of serotonergic projections. J Histochem Cytochem *30*, 805-810.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*, 248-254.

Brodie, T. G. (1900). The immediate action of an intravenous injection of blood serum. J Physiol *26*, 48-71.

Broening, H. W., Bowyer, J. F., and Slikker, W., Jr. (1995). Age-dependent sensitivity of rats to the long-term effects of the serotonergic neurotoxicant (+/-)-3,4-methylenedioxymethamphetamine (MDMA) correlates with the magnitude of the MDMA-induced thermal response. J Pharmacol Exp Ther *275*, 325-333.

Bruning, G., and Liangos, O. (1997). Transient expression of the serotonin transporter in the developing mouse thalamocortical system. Acta Histochem *99*, 117-121.

Bruning, G., Liangos, O., and Baumgarten, H. G. (1997). Prenatal development of the serotonin transporter in mouse brain. Cell Tissue Res *289*, 211-221.

Bryer, J. B., Starkstein, S. E., Votypka, V., Parikh, R. M., Price, T. R., and Robinson, R. G. (1992). Reduction of CSF monoamine metabolites in poststroke depression: a preliminary report. J Neuropsychiatry Clin Neurosci *4*, 440-442.

Buhot, M. C., Martin, S., and Segu, L. (2000). Role of serotonin in memory impairment. Ann Med *32*, 210-221.

Burgess, C., O'Donohoe, A., and Gill, M. (2000). Agony and ecstasy: a review of MDMA effects and toxicity. Eur Psychiatry *15*, 287-294.

Calogero, A. E., Bagdy, G., Szemeredi, K., Tartaglia, M. E., Gold, P. W., and Chrousos, G. P. (1990). Mechanisms of serotonin receptor agonist-induced activation of the hypothalamic-pituitary-adrenal axis in the rat. Endocrinology *126*, 1888-1894.

Cao, Y., Li, M., Mager, S., and Lester, H. A. (1998). Amino acid residues that control pH modulation of transport-associated current in mammalian serotonin transporters. J Neurosci *18*, 7739-7749.

Carlsson, A. (1969). Pharmacology of synaptic monoamine transmission. Prog Brain Res *31*, 53-59.

Carlsson, A., Corrodi, H., Fuxe, K., and Hokfelt, T. (1969). Effect of antidepressant drugs on the depletion of intraneuronal brain 5-Hydroxytryptamine stores caused by 4-methyl-alpha-ethyl-meta-tyramine. Eur J Pharmacol *5*, 357-366.

Carlsson, A., Fuxe, K., and Ungerstedt, U. (1968). The effect of imipramine on central 5-Hydroxytryptamine neurons. J Pharm Pharmacol *20*, 150-151.

Carlsson, A., and Lindqvist, M. (1978). Effects of antidepressant agents on the synthesis of brain monoamines. J Neural Transm *43*, 73-91.

Cases, O., Lebrand, C., Giros, B., Vitalis, T., De Maeyer, E., Caron, M. G., Price, D. J., Gaspar, P., and Seif, I. (1998). Plasma membrane transporters of serotonin, dopamine, and norepinephrine mediate serotonin accumulation in atypical locations in the developing brain of monoamine oxidase A knock-outs. J Neurosci *18*, 6914-6927.

Cases, O., Seif, I., Grimsby, J., Gaspar, P., Chen, K., Pournin, S., Muller, U., Aguet, M., Babinet, C., Shih, J. C., and et al. (1995). Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. Science *268*, 1763-1766.

Cases, O., Vitalis, T., Seif, I., De Maeyer, E., Sotelo, C., and Gaspar, P. (1996). Lack of barrels in the somatosensory cortex of monoamine oxidase A- deficient mice: role of a serotonin excess during the critical period. Neuron *16*, 297-307.

Celada, P., Perez, J., Alvarez, E., and Artigas, F. (1992). Monoamine oxidase inhibitors phenelzine and brofaromine increase plasma serotonin and decrease 5-Hydroxyindoleacetic acid in patients with major depression: relationship to clinical improvement. J Clin Psychopharmacol *12*, 309-315.

Chanda, P. K., Minchin, M. C., Davis, A. R., Greenberg, L., Reilly, Y., McGregor, W. H., Bhat, R., Lubeck, M. D., Mizutani, S., and Hung, P. P. (1993). Identification of residues important for ligand binding to the human 5- hydroxytryptamine1A serotonin receptor. Mol Pharmacol *43*, 516-520.

Cheifetz, S., and Warsh, J. J. (1980). Occurrence and distribution of 5-Hydroxytryptophol in the rat. J Neurochem *34*, 1093-1099.

Chen, J. J., Li, Z., Pan, H., Murphy, D. L., Tamir, H., Koepsell, H., and Gershon, M. D. (2001). Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: Abnormal intestinal motility and the expression of cation transporters. J Neurosci *21*, 6348-6361.

Cheng, Y., and Prusoff, W. H. (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol *22*, 3099-3108.

Colado, M. I., Granados, R., O'Shea, E., Esteban, B., and Green, A. R. (1998). Role of hyperthermia in the protective action of clomethiazole against MDMA ('ecstasy')-induced neurodegeneration, comparison with the novel NMDA channel blocker AR-R15896AR. Br J Pharmacol *124*, 479-484.

Colado, M. I., Murray, T. K., and Green, A. R. (1993). 5-HT loss in rat brain following 3,4-methylenedioxymethamphetamine (MDMA), p-chloroamphetamine and fenfluramine administration and effects of chlormethiazole and dizocilpine. Br J Pharmacol *108*, 583-589.

Collier, D. A., Stober, G., Li, T., Heils, A., Catalano, M., Di Bella, D., Arranz, M. J., Murray, R. M., Vallada, H. P., Bengel, D., *et al.* (1996). A novel functional polymorphism within the promoter of the serotonin transporter gene: possible role in susceptibility to affective disorders. Mol Psychiatry *1*, 453-460.

Cool, D. R., Leibach, F. H., Bhalla, V. K., Mahesh, V. B., and Ganapathy, V. (1991). Expression and cyclic AMP-dependent regulation of a high affinity serotonin transporter in the human placental choriocarcinoma cell line (JAR). J Biol Chem *266*, 15750-15757.

Coppen, A., Shaw, D. M., Herzberg, B., and Maggs, R. (1967). Tryptophan in the treatment of depression. Lancet *2*, 1178-1180.

Corrodi, H., and Fuxe, K. (1969). Decreased turnover in central 5-HT nerve terminals induced by antidepressant drugs of the imipramine type. Eur J Pharmacol *7*, 56-59.

Costentin, J., Duterte-Boucher, D., Panissaud, C., and Michael-Titus, A. (1990). Dopamine D1 and D2 receptors mediate opposite effects of apomorphine on the body temperature of reserpinized mice. Neuropharmacology *29*, 31-35.

Cox, B., Kerwin, R. W., Lee, T. F., and Pycock, C. J. (1980). A dopamine-5-Hydroxytryptamine link in the hypothalamic pathways which mediate heat loss in the rat. J Physiol *303*, 9-21.

Crawshaw, L. I. (1972). Effects of intracerebral 5-Hydroxytryptamine injection on themoregulation in rat. Physiol Behav *9*, 133-140.

Crespi, D., Mennini, T., and Gobbi, M. (1997). Carrier-dependent and Ca(2+)-dependent 5-HT and dopamine release induced by (+)-amphetamine, 3,4-methylendioxymethamphetamine, p- chloroamphetamine and (+)-fenfluramine. Br J Pharmacol *121*, 1735-1743.

Curzon, G., and Gibson, E. L. (1999). The serotonergic appetite suppressant fenfluramine. Reappraisal and rejection. Adv Exp Med Biol *467*, 95-100.

Dafters RI, L. E. (1998). Persistent loss of thermoregulation in the rat induced by 3,4-methylenedioxymethamphetamine (MDMA or "Ecstasy") but not by fenfluramine. Psychopharmacology (Berl) *138*, 207-212.

Dahlstrom, A., and Fuxe, K. (1964). Localization of monoamines in the lower brain stem. Experientia *20*, 398-399.

Dahlstrom, A., Fuxe, K., Kernell, D., and Sedvall, G. (1965). Reduction of the monoamine stores in the terminals of bulbospinal neurones following stimulation in the medulla oblongata. Life Sci *4*, 1207-1212.

Dahlstrom, M., Ahonen, A., Ebeling, H., Torniainen, P., Heikkila, J., and Moilanen, I. (2000). Elevated hypothalamic/midbrain serotonin (monoamine) transporter availability in depressive drug-naive children and adolescents. Mol Psychiatry *5*, 514-522.

Dar, K. J., and McBrien, M. E. (1996). MDMA induced hyperthermia: report of a fatality and review of current therapy. Intensive Care Med *22*, 995-996.

Davis, L. G., Arentzen, R., Reid, J. M., Manning, R. W., Wolfson, B., Lawrence, K. L., and Baldino, F., Jr. (1986). Glucocorticoid sensitivity of vasopressin mRNA levels in the paraventricular nucleus of the rat. Proc Natl Acad Sci U S A *83*, 1145-1149.

Daws, L. C., Gould, G. G., Teicher, S. D., Gerhardt, G. A., and Frazer, A. (2000). 5-HT(1B) receptor-mediated regulation of serotonin clearance in rat hippocampus in vivo. J Neurochem *75*, 2113-2122.

Dawson, L. A., Nguyen, H. Q., Smith, D. I., and Schechter, L. E. (2000). Effects of chronic fluoxetine treatment in the presence and absence of (+/-)pindolol: a microdialysis study. Br J Pharmacol *130*, 797-804.

de Roij, T. A., Frens, J., Vianen-Meijerink, M., and Nijnanten, F. W. (1979). Relation between the thermoregulatory effects of intracerebroventricularly injected dopamine and 5-Hydroxytryptamine in the rabbit. Naunyn Schmiedebergs Arch Pharmacol *306*, 61-66.

Delgado, P. L. (2000). Depression: the case for a monoamine deficiency. J Clin Psychiatry *61*, 7-11.

Delgado, P. L., Charney, D. S., Price, L. H., Aghajanian, G. K., Landis, H., and Heninger, G. R. (1990). Serotonin function and the mechanism of antidepressant action. Reversal of antidepressant-induced remission by rapid depletion of plasma tryptophan. Arch Gen Psychiatry *47*, 411-418.

Dencker, S. J., Malm, U., Roos, B. E., and Werdinius, B. (1966). Acid monoamine metabolites of cerebrospinal fluid in mental depression and mania. J Neurochem *13*, 1545-1548.

Di Matteo, V., Di Giovanni, G., Di Mascio, M., and Esposito, E. (1998). Selective blockade of serotonin2C/2B receptors enhances dopamine release in the rat nucleus accumbens. Neuropharmacology *37*, 265-272.

Duncan, R. J., and Sourkes, T. L. (1974). Some enzymic aspects of the production of oxidized or reduced metabolites of catecholamines and 5-Hydroxytryptamine by brain tissues. J Neurochem *22*, 663-669.

Engel, S. R., and Allan, A. M. (1999). 5-HT3 receptor over-expression enhances ethanol sensitivity in mice. Psychopharmacology (Berl) *144*, 411-415.

Engel, S. R., Lyons, C. R., and Allan, A. M. (1998). 5-HT3 receptor over-expression decreases ethanol self administration in transgenic mice. Psychopharmacology (Berl) *140*, 243-248.

Erspamer, V. (1963). 5-Hydroxytryptamine. In Comparative Endocrinolgy, U. S. von Euler, Heller, H., ed., pp. 159-181.

Fabre, V., Beaufour, C., Evrard, A., Rioux, A., Hanoun, N., Lesch, K. P., Murphy, D. L., Lanfumey, L., Hamon, M., and Martres, M. P. (2000). Altered expression and functions of serotonin 5-HT1A and 5-HT1B receptors in knock-out mice lacking the 5-HT transporter. Eur J Neurosci *12*, 2299-2310.

Feldberg, W. (1969). The role of monoamines in the hypothalamus for temperature regulation. J Neurovisc Relat *31*, 362+.

Feldstein, A., and Williamson, O. (1968). Serotonin metabolism in pineal homogenates. Adv Pharmacol *6*, 91-96.

Fischer, H. S., Zernig, G., Schatz, D. S., Humpel, C., and Saria, A. (2000). MDMA ('ecstasy') enhances basal acetylcholine release in brain slices of the rat striatum. Eur J Neurosci *12*, 1385-1390.

Foguet, M., Hartikka, J. A., Schmuck, K., and Lubbert, H. (1993). Long-term regulation of serotonergic activity in the rat brain via activation of protein kinase A. Embo J *12*, 903-910.

Franklin, K., Paxinos, G (1997). The Mouse Brain in Stereotaxic Coordinates, 1st edn (San Diego,, Academic Press).

Fuller, R. W., Perry, K. W., and Molloy, B. B. (1974). Effect of an uptake inhibitor on serotonin metabolism in rat brain: studies with 3-(p-trifluoromethylphenoxy)-N-methyl-3-phenylpropylamine (Lilly 110140). Life Sci *15*, 1161-1171.

Galli, A., Blakely, R. D., and DeFelice, L. J. (1996). Norepinephrine transporters have channel modes of conduction. Proc Natl Acad Sci U S A *93*, 8671-8676.

Galli, A., DeFelice, L. J., Duke, B. J., Moore, K. R., and Blakely, R. D. (1995). Sodium-dependent norepinephrine-induced currents in norepinephrine- transporter-transfected HEK-293 cells blocked by cocaine and antidepressants. J Exp Biol *198*, 2197-2212.

Galli, A., Petersen, C. I., deBlaquiere, M., Blakely, R. D., and DeFelice, L. J. (1997). Drosophila serotonin transporters have voltage-dependent uptake coupled to a serotonin-gated ion channel. J Neurosci *17*, 3401-3411.

Garattini, S., Mennini, T., Bendotti, C., Invernizzi, R., and Samanin, R. (1986). Neurochemical mechanism of action of drugs which modify feeding via the serotoninergic system. Appetite *7*, 15-38.

Gartside, S. E., McQuade, R., and Sharp, T. (1997). Acute effects of 3,4-methylenedioxymethamphetamine (MDMA) on 5-HT cell firing and release: comparison between dorsal and median raphe 5-HT systems. Neuropharmacology *36*, 1697-1703.

Gartside, S. E., Umbers, V., Hajos, M., and Sharp, T. (1995). Interaction between a selective 5-HT1A receptor antagonist and an SSRI in vivo: effects on 5-HT cell firing and extracellular 5-HT. Br J Pharmacol *115*, 1064-1070.

Giraldo, P., and Montoliu, L. (2001). Size matters: use of YACs, BACs and PACs in transgenic animals. Transgenic Res *10*, 83-103.

Gobbi, G., Murphy, D. L., Lesch, K., and Blier, P. (2001). Modifications of the serotonergic system in mice lacking serotonin transporters: an in vivo electrophysiological study. J Pharmacol Exp Ther *296*, 987-995.

Gold, L. H., Koob, G. F., and Geyer, M. A. (1988). Stimulant and hallucinogenic behavioral profiles of 3,4- methylenedioxymethamphetamine and N-ethyl-3,4-methylenedioxyamphetamine in rats. J Pharmacol Exp Ther *247*, 547-555.

Goodwin, G. M., De Souza, R. J., and Green, A. R. (1985). The pharmacology of the hypothermic response in mice to 8-hydroxy-2-(di- n-propylamino)tetralin (8-OH-DPAT). A model of presynaptic 5-HT1 function. Neuropharmacology *24*, 1187-1194.

Gordon, J. L., and Olverman, H. J. (1978). 5-Hydroxytryptamine and dopamine transport by rat and human blood platelets. Br JPharmacol *62*, 219-226.

Graham, D., Esnaud, H., Habert, E., and Langer, S. Z. (1989). A common binding site for tricyclic and nontricyclic 5- hydroxytryptamine uptake inhibitors at the substrate recognition site of the neuronal sodium-dependent 5-Hydroxytryptamine transporter. Biochem Pharmacol *38*, 3819-3826.

Grahame-Smith, D. G. (1964). Tryptophan hydroxylation in brain. Biochem Biophys Res Commun *16*, 586-592.

Gu, X. F., and Azmitia, E. C. (1993). Integrative transporter-mediated release from cytoplasmic and vesicular 5-Hydroxytryptamine stores in cultured neurons. Eur J Pharmacol *235*, 51-57.

Gudelsky, G. A., Koenig, J. I., and Meltzer, H. Y. (1986). Thermoregulatory responses to serotonin (5-HT) receptor stimulation in the rat. Evidence for opposing roles of 5-HT2 and 5-HT1A receptors. Neuropharmacology *25*, 1307-1313.

Gudelsky, G. A., and Nash, J. F. (1996). Carrier-mediated release of serotonin by 3,4- methylenedioxymethamphetamine: implications for serotonin-dopamine interactions. J Neurochem *66*, 243-249.

Hall, F. S., Li, X. F., Sora, I., Xu, F., Caron, M., Lesch, K. P., Murphy, D. L., and Uhl, G. R. (2002). Cocaine mechanisms: enhanced cocaine, fluoxetine and nisoxetine place preferences following monoamine transporter deletions. Neuroscience *115*, 153-161.

Hamon, M., Bourgoin, S., Artaud, F., and El Mestikawy, S. (1981). The respective roles of tryptophan uptake and tryptophan hydroxylase in the regulation of serotonin synthesis in the central nervous system. J Physiol *77*, 269-279.

Hamon, M., and Glowinski, J. (1974). Regulation of serotonin synthesis. Life Sci *15*, 1533-1548.

Hansson, S. R., Mezey, E., and Hoffman, B. J. (1998). Serotonin transporter messenger RNA in the developing rat brain: early expression in serotonergic neurons and transient expression in non- serotonergic neurons. Neuroscience *83*, 1185-1201.

Hansson, S. R., Mezey, E., and Hoffman, B. J. (1999). Serotonin transporter messenger RNA expression in neural crest-derived structures and sensory pathways of the developing rat embryo. Neuroscience *89*, 243-265.

Harmar, A. J., Ogilvie, A. D., Battersby, S., Smith, C. A., Blackwood, D. H., Muir, W. J., Fink, G., and Goodwin, G. M. (1996). The serotonin transporter gene and affective disorder. Cold Spring Harb Symp Quant Biol *61*, 791-795.

Hebert, C., Habimana, A., Elie, R., and Reader, T. A. (2001). Effects of chronic antidepressant treatments on 5-HT and NA transporters in rat brain: an autoradiographic study. Neurochem Int *38*, 63-74.

Heils, A., Teufel, A., Petri, S., Seemann, M., Bengel, D., Balling, U., Riederer, P., and Lesch, K. P. (1995). Functional promoter and polyadenylation site mapping of the human serotonin (5-HT) transporter gene. J Neural Transm Gen Sect *102*, 247-254.

Heils, A., Teufel, A., Petri, S., Stober, G., Riederer, P., Bengel, D., and Lesch, K. P. (1996). Allelic variation of human serotonin transporter gene expression. J Neurochem *66*, 2621-2624.

Hernandez, L., Parada, M., Baptista, T., Schwartz, D., West, H. L., Mark, G. P., and Hoebel, B. G. (1991). Hypothalamic serotonin in treatments for feeding disorders and depression as studied by brain microdialysis. J Clin Psychiatry *52 Suppl*, 32-40.

Hertz, D., and Sulman, F. G. (1968). Preventing depression with tryptophan. Lancet *1*, 531-532.

Hilakivi, I. (1987). Biogenic amines in the regulation of wakefulness and sleep. Med Biol *65*, 97-104.

Hirata, H., Negoro, S., and Okada, H. (1984). Molecular basis of isozyme formation of beta-galactosidases in Bacillus stearothermophilus: isolation of two beta-galactosidase genes, bgaA and bgaB. J Bacteriol *160*, 9-14.

Hjorth, S. (1985). Hypothermia in the rat induced by the potent serotoninergic agent 8-OH- DPAT. J Neural Transm *61*, 131-135.

Hjorth, S. (1993). Serotonin 5-HT1A autoreceptor blockade potentiates the ability of the 5- HT reuptake inhibitor citalopram to increase nerve terminal output of 5- HT in vivo: a microdialysis study. J Neurochem *60*, 776-779.

Hjorth, S., and Sharp, T. (1993). In vivo microdialysis evidence for central serotonin1A and serotonin1B autoreceptor blocking properties of the beta adrenoceptor antagonist (-)penbutolol. J Pharmacol Exp Ther *265*, 707-712.

Hjorth, S., Suchowski, C. S., and Galloway, M. P. (1995). Evidence for 5-HT autoreceptor-mediated, nerve impulse-independent, control of 5-HT synthesis in the rat brain. Synapse *19*, 170-176.

Hoehe, M. R., Wendel, B., Grunewald, I., Chiaroni, P., Levy, N., Morris-Rosendahl, D., Macher, J. P., Sander, T., and Crocq, M. A. (1998). Serotonin transporter (5-HTT) gene polymorphisms are not associated with susceptibility to mood disorders. Am J Med Genet *81*, 1-3.

Hoffman, B. J., Mezey, E., and Brownstein, M. J. (1991). Cloning of a serotonin transporter affected by antidepressants. Science *254*, 579-580.

Invernizzi, R., Belli, S., and Samanin, R. (1992). Citalopram's ability to increase the extracellular concentrations of serotonin in the dorsal raphe prevents the drug's effect in the frontal cortex. Brain Res *584*, 322-324.

Irwin, S. (1968). Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia *13*, 222-257.

Ivgy-May, N., Tamir, H., and Gershon, M. D. (1994). Synaptic properties of serotonergic growth cones in developing rat brain. J Neurosci *14*, 1011-1029.

Jacobs, B. L., and Azmitia, E. C. (1992). Structure and function of the brain serotonin system. Physiol Rev *72*, 165-229.

Jacobson, R. H., Zhang, X. J., DuBose, R. F., and Matthews, B. W. (1994). Three-dimensional structure of beta-galactosidase from E. coli. Nature *369*, 761-766.

Jayanthi, L. D., Ramamoorthy, S., Mahesh, V. B., Leibach, F. H., and Ganapathy, V. (1994). Calmodulin-dependent regulation of the catalytic function of the human serotonin transporter in placental choriocarcinoma cells. J Biol Chem *269*, 14424-14429.

Joh, T. H. (1998). Tryptophan Hydroxylase: Molecular Biology and Regulation. In Serotonergic Neurons and 5-HT receptors in the CNS, H. G. a. G. Baumgarten, M., ed. (Springer), pp. 117-126.

Joh, T. H., Shikimi, T., Pickel, V. M., and Reis, D. J. (1975). Brain tryptophan hydroxylase: purification of, production of antibodies to, and cellular and ultrastructural localization in serotonergic neurons of rat midbrain. Proc Natl Acad Sci U S A *72*, 3575-3579.

Johnston, J. P. (1968). Some observations upon a new inhibitor of monoamine oxidase in brain tissue. Biochem Pharmacol *17*, 1285-1297.

Jonsson, E. G., Norton, N., Gustavsson, J. P., Oreland, L., Owen, M. J., and Sedvall, G. C. (2000). A promoter polymorphism in the monoamine oxidase A gene and its relationships to monoamine metabolite concentrations in CSF of healthy volunteers. J Psychiatr Res *34*, 239-244.

Juers, D. H., Jacobson, R. H., Wigley, D., Zhang, X. J., Huber, R. E., Tronrud, D. E., and Matthews, B. W. (2000). High resolution refinement of beta-galactosidase in a new crystal form reveals multiple metal-binding sites and provides a structural basis for alpha-complementation. Protein Sci *9*, 1685-1699.

Kaneshiro, C. M., Enns, C. A., Hahn, M. G., Peterson, J. S., and Reithel, F. J. (1975). Evidence for an active dimer of Escherichia coli beta-galactosidase. Biochem J *151*, 433-434.

Kanner, B. I., and Schuldiner, S. (1987). Mechanism of transport and storage of neurotransmitters. CRC Crit Rev Biochem *22*, 1-38.

Kaye, W., Gendall, K., and Strober, M. (1998). Serotonin neuronal function and selective serotonin reuptake inhibitor treatment in anorexia and bulimia nervosa. Biol Psychiatry *44*, 825-838.

Klein, D. C., Weller, J. L., and Moore, R. Y. (1971). Melatonin metabolism: neural regulation of pineal serotonin: acetyl coenzyme A N-acetyltransferase activity. Proc Natl Acad Sci U S A *68*, 3107-3110.

Koob, G. F. (2000). Neurobiology of addiction. Toward the development of new therapies. Ann N Y Acad Sci *909*, 170-185.

Kosofsky, B. E., and Molliver, M. E. (1987). The serotoninergic innervation of cerebral cortex: different classes of axon terminals arise from dorsal and median raphe nuclei. Synapse *1*, 153-168.

Kuhn, D. M., and Lovenberg, W. (1982). Role of calmodulin in the activation of tryptophan hydroxylase. Fed Proc *41*, 2258-2264.

Kunugi, H., Hattori, M., Kato, T., Tatsumi, M., Sakai, T., Sasaki, T., Hirose, T., and Nanko, S. (1997). Serotonin transporter gene polymorphisms: ethnic difference and possible association with bipolar affective disorder. Mol Psychiatry *2*, 457-462.

la Cour, C. M., Boni, C., Hanoun, N., Lesch, K. P., Hamon, M., and Lanfumey, L. (2001). Functional consequences of 5-HT transporter gene disruption on 5-HT(1a) receptor-mediated regulation of dorsal raphe and hippocampal cell activity. J Neurosci *21*, 2178-2185.

Lapin, I. P., and Oxenkrug, G. F. (1969). Intensification of the central serotoninergic processes as a possible determinant of the thymoleptic effect. Lancet *1*, 132-136.

Larsson, L. G., Stenfors, C., and Ross, S. B. (1998). Differential regional antagonism of 8-OH-DPAT-induced decrease in serotonin synthesis by two 5-HT1A receptor antagonists. Eur J Pharmacol *346*, 209-215.

Lauder, J. M. (1990). Ontogeny of the serotonergic system in the rat: serotonin as a developmental signal. Ann N Y Acad Sci *600*, 297-313.

Lauder, J. M. (1993). Neurotransmitters as growth regulatory signals: role of receptors and second messengers. Trends Neurosci *16*, 233-240.

Lauder, J. M., Liu, J., and Grayson, D. R. (2000). In utero exposure to serotonergic drugs alters neonatal expression of 5- HT(1A) receptor transcripts: a quantitative RT-PCR study. Int J Dev Neurosci *18*, 171-176.

Lauder, J. M., Wallace, J. A., Wilkie, M. B., DiNome, A., and Krebs, H. (1983). Roles for serotonin in neurogenesis. Monogr Neural Sci *9*, 3-10.

Launay, J. M., Bondoux, D., Oset-Gasque, M. J., Emami, S., Mutel, V., Haimart, M., and Gespach, C. (1994). Increase of human platelet serotonin uptake by atypical histamine receptors. Am J Physiol *266*, R526-536.

Lawrence, J. A., Olverman, H. J., Shirakawa, K., Kelly, J. S., and Butcher, S. P. (1993). Binding of 5-HT1A receptor and 5-HT transporter ligands in rat cortex and hippocampus following cholinergic and serotonergic lesions. Brain Res *612*, 326-329.

Le Poul, E., Boni, C., Hanoun, N., Laporte, A. M., Laaris, N., Chauveau, J., Hamon, M., and Lanfumey, L. (2000). Differential adaptation of brain 5-HT1A and 5-HT1B receptors and 5-HT transporter in rats treated chronically with fluoxetine. Neuropharmacology *39*, 110-122.

Leibowitz, S. F., and Alexander, J. T. (1998). Hypothalamic serotonin in control of eating behavior, meal size, and body weight. Biol Psychiatry *44*, 851-864.

Leroux-Nicollet, I., Panissaud, C., and Costentin, J. (1988). Involvement of norepinephrine neurons in the hypothermia induced by intracerebroventricular administration of 6-hydroxydopamine in mice, evidenced by antidepressants. J Neural Transm *74*, 17-27.

Lesch, K. P., Aulakh, C. S., Wolozin, B. L., Tolliver, T. J., Hill, J. L., and Murphy, D. L. (1993). Regional brain expression of serotonin transporter mRNA and its regulation by reuptake inhibiting antidepressants. Brain Res Mol Brain Res *17*, 31-35.

Lesch, K. P., Bengel, D., Heils, A., Sabol, S. Z., Greenberg, B. D., Petri, S., Benjamin, J., Muller, C. R., Hamer, D. H., and Murphy, D. L. (1996). Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science *274*, 1527-1531.

Lester, H. A., Cao, Y., and Mager, S. (1996). Listening to neurotransmitter transporters. Neuron *17*, 807-810.

Lester, H. A., Mager, S., Quick, M. W., and Corey, J. L. (1994). Permeation properties of neurotransmitter transporters. Annu Rev Pharmacol Toxicol *34*, 219-249.

Levi, G., and Raiteri, M. (1993). Carrier-mediated release of neurotransmitters. Trends Neurosci *16*, 415-419.

Li, Q., Wichems, C., Heils, A., Lesch, K. P., and Murphy, D. L. (2000). Reduction in the density and expression, but not G-protein coupling, of serotonin receptors (5-HT1A) in 5-HT transporter knock-out mice: gender and brain region differences. J Neurosci *20*, 7888-7895.

Li, Q., Wichems, C., Heils, A., Van De Kar, L. D., Lesch, K. P., and Murphy, D. L. (1999a). Reduction of 5-Hydroxytryptamine (5-HT)(1A)-mediated temperature and neuroendocrine responses and 5-HT(1A) binding sites in 5-HT transporter knockout mice. J Pharmacol Exp Ther *291*, 999-1007.

Li, X. M., Perry, K. W., and Wong, D. T. (1999b). Difference in the in vivo influence of serotonin1A autoreceptors on serotonin release in prefrontal cortex and dorsal hippocampus of the same rat treated with fluoxetine. Chin J Physiol *42*, 53-59.

Lin, F., Lester, H. A., and Mager, S. (1996). Single-channel currents produced by the serotonin transporter and analysis of a mutation affecting ion permeation. Biophys J *71*, 3126-3135.

Lin, M., Pang, IH,Chern,SI,Chia,WY (1978). Changes in serotonin contents in brain affect metabolic heat production of rabbits in cold. Am J Physiol *235*, R41-47.

Lin, M. T., Chandra, A., Ko, W. C., and Chen, Y. M. (1981). Serotonergic mechanisms of clonidine-induced hypothermia in rats. Neuropharmacology *20*, 15-21.

Lin, M. T., Tsay, H. J., Su, W. H., and Chueh, F. Y. (1998). Changes in extracellular serotonin in rat hypothalamus affect thermoregulatory function. Am J Physiol *274*, R1260-1267.

Lin, M. T., Wu, J. J., and Tsay, B. L. (1983). Serotonergic mechanisms in the hypothalamus mediate thermoregulatory responses in rats. Naunyn Schmiedebergs Arch Pharmacol *322*, 271-278.

Liu, Y., and Edwards, R. H. (1997). The role of vesicular transport proteins in synaptic transmission and neural degeneration. Annu Rev Neurosci *20*, 125-156.

Lloyd, K. G., Farley, I. J., Deck, J. H., and Hornykiewicz, O. (1974). Serotonin and 5-Hydroxyindoleacetic acid in discrete areas of the brainstem of suicide victims and control patients. Adv Biochem Psychopharmacol *11*, 387-397.

Logan, B., Laverty, R,Sanderson, W,Ben Lee, Y (1988). Differences between rats and mice in MDMA (methylenedioxymethamphetamine) neurotoxicity. Eur J Pharm *152*, 227-234.

Lopez, J. F., Vazquez, D. M., Chalmers, D. T., and Watson, S. J. (1997). Regulation of 5-HT receptors and the hypothalamic-pituitary-adrenal axis. Implications for the neurobiology of suicide. Ann N Y Acad Sci *836*, 106-134.

Lorang, D., Amara, S. G., and Simerly, R. B. (1994). Cell-type-specific expression of catecholamine transporters in the rat brain. J Neurosci *14*, 4903-4914.

Mager, S., Min, C., Henry, D. J., Chavkin, C., Hoffman, B. J., Davidson, N., and Lester, H. A. (1994). Conducting states of a mammalian serotonin transporter. Neuron *12*, 845-859.

Malagie, I., Trillat, A. C., Douvier, E., Anmella, M. C., Dessalles, M. C., Jacquot, C., and Gardier, A. M. (1996). Regional differences in the effect of the combined treatment of WAY 100635 and fluoxetine: an in vivo microdialysis study. Naunyn Schmiedebergs Arch Pharmacol *354*, 785-790.

Malagie, I., Trillat, A. C., Jacquot, C., and Gardier, A. M. (1995). Effects of acute fluoxetine on extracellular serotonin levels in the raphe: an in vivo microdialysis study. Eur J Pharmacol *286*, 213-217.

Malberg, J. E., Sabol, K. E., and Seiden, L. S. (1996). Co-administration of MDMA with drugs that protect against MDMA neurotoxicity produces different effects on body temperature in the rat. J Pharmacol Exp Ther *278*, 258-267.

Malison, R. T., Price, L. H., Berman, R., van Dyck, C. H., Pelton, G. H., Carpenter, L., Sanacora, G., Owens, M. J., Nemeroff, C. B., Rajeevan, N., *et al.* (1998). Reduced brain serotonin transporter availability in major depression as measured by [123I]-2 beta-carbomethoxy-3 beta-(4-iodophenyl)tropane and single photon emission computed tomography. Biol Psychiatry *44*, 1090-1098.

Mallick, A., and Bodenham, A. R. (1997). MDMA induced hyperthermia: a survivor with an initial body temperature of 42.9 degrees C. J Accid Emerg Med *14*, 336-338.

Mamounas, L. A., and Molliver, M. E. (1988). Evidence for dual serotonergic projections to neocortex: axons from the dorsal and median raphe nuclei are differentially vulnerable to the neurotoxin p-chloroamphetamine (PCA). Exp Neurol *102*, 23-36.

Mann, J. J., Huang, Y. Y., Underwood, M. D., Kassir, S. A., Oppenheim, S., Kelly, T. M., Dwork, A. J., and Arango, V. (2000). A serotonin transporter gene promoter polymorphism (5-HTTLPR) and prefrontal cortical binding in major depression and suicide. Arch Gen Psychiatry *57*, 729-738.

Maricq, A. V., Peterson, A. S., Brake, A. J., Myers, R. M., and Julius, D. (1991). Primary structure and functional expression of the 5HT3 receptor, a serotonin-gated ion channel. Science *254*, 432-437.

Martensson, B., Nyberg, S., Toresson, G., Brodin, E., and Bertilsson, L. (1989). Fluoxetine treatment of depression. Clinical effects, drug concentrations and monoamine metabolites and N-terminally extended substance P in cerebrospinal fluid. Acta Psychiatr Scand *79*, 586-596.

Masson, J., Riad, M., Chaudhry, F., Darmon, M., Aidouni, Z., Conrath, M., Giros, B., Hamon, M., Storm-Mathisen, J., Descarries, L., and El Mestikawy, S. (1999). Unexpected localization of the Na+/Cl--dependent-like orphan transporter, Rxt1, on synaptic vesicles in the rat central nervous system. Eur J Neurosci *11*, 1349-1361.

Maura, G., Gemignani, A., and Raiteri, M. (1982). Noradrenaline inhibits central serotonin release through alpha 2- adrenoceptors located on serotonergic nerve terminals. Naunyn Schmiedebergs Arch Pharmacol *320*, 272-274.

McAllister-Williams, R. H., Anderson, A. J., and Young, A. H. (2001). Corticosterone selectively attenuates 8-OH-DPAT-mediated hypothermia in mice. Int J Neuropsychopharmacol *4*, 1-8.

McQueen, J. K., Wilson, H., and Fink, G. (1997). Estradiol-17 beta increases serotonin transporter (SERT) mRNA levels and the density of SERT-binding sites in female rat brain [In Process Citation]. Brain Res Mol Brain Res *45*, 13-23.

Meguid, M. M., Fetissov, S. O., Varma, M., Sato, T., Zhang, L., Laviano, A., and Rossi-Fanelli, F. (2000). Hypothalamic dopamine and serotonin in the regulation of food intake. Nutrition *16*, 843-857.

Millan, M. J., Dekeyne, A., and Gobert, A. (1998). Serotonin (5-HT)2C receptors tonically inhibit dopamine (DA) and noradrenaline (NA), but not 5-HT, release in the frontal cortex in vivo. Neuropharmacology *37*, 953-955.

Miller D, O. C. J. (1994). enviroment-, Drug- and Stress-Induced Alterations in body temperature Affect the neurotoxicity of substituted Amphetamines in the C57Bl/6J Mouse. JPET *270*, 752-760.

Miller, D. B., and O'Callaghan, J. P. (1995). The role of temperature, stress, and other factors in the neurotoxicity of the substituted amphetamines 3,4-methylenedioxymethamphetamine and fenfluramine. Mol Neurobiol *11*, 177-192.

Miller, K. J., and Hoffman, B. J. (1994). Adenosine A3 receptors regulate serotonin transport via nitric oxide and cGMP. J Biol Chem *269*, 27351-27356.

Moiseiwitsch, J. R., and Lauder, J. M. (1995). Serotonin regulates mouse cranial neural crest migration. Proc Natl Acad Sci U S A *92*, 7182-7186.

Molliver, M. E. (1987). Serotonergic neuronal systems: what their anatomic organization tells us about function. J Clin Psychopharmacol *7*, 3S-23S.

Monti, J. M., and Monti, D. (2000). Role of dorsal raphe nucleus serotonin 5-HT1A receptor in the regulation of REM sleep. Life Sci *66*, 1999-2012.

Moret, C., and Briley, M. (1997a). 5-HT autoreceptors in the regulation of 5-HT release from guinea pig raphe nucleus and hypothalamus. Neuropharmacology *36*, 1713-1723.

Moret, C., and Briley, M. (1997b). Ex vivo inhibitory effect of the 5-HT uptake blocker citalopram on 5-HT synthesis. J Neural Transm *104*, 147-160.

Morilak, D. A., and Ciaranello, R. D. (1993). Ontogeny of 5-Hydroxytryptamine2 receptor immunoreactivity in the developing rat brain. Neuroscience *55*, 869-880.

Morin, L. P. (1999). Serotonin and the regulation of mammalian circadian rhythmicity. Ann Med *31*, 12-33.

Morishima, Y., and Shibano, T. (1995). Evidence that 5-HT2A receptors are not involved in 5-HT-mediated thermoregulation in mice. Pharmacol Biochem Behav *52*, 755-758.

Mossner, R., Albert, D., Persico, A. M., Hennig, T., Bengel, D., Holtmann, B., Schmitt, A., Keller, F., Simantov, R., Murphy, D., *et al.* (2000). Differential regulation of adenosine A(1) and A(2A) receptors in serotonin transporter and monoamine oxidase A-deficient mice. Eur Neuropsychopharmacol *10*, 489-493.

Mountford P, Z. B., Duwel A, Nichols J, Li M, Dani C, Robertson M, Chambers I, Smith A. (1994). Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. Proceedings of the National Academy of Sciences *91*, 4303-4307.

Myers, C. L., Lazo, J. S., and Pitt, B. R. (1989). Translocation of protein kinase C is associated with inhibition of 5-HT uptake by cultured endothelial cells. Am J Physiol *257*, L253-258.

Myers, R. D., and Beleslin, D. B. (1971). Changes in serotonin release in hypothalamus during cooling or warming of the monkey. Am J Physiol *220*, 1746-1754.

Myers, R. D., and Waller, M. B. (1975). 5-HT-and norepinephrine-induced release of ACh from the thalamus and mesencephalon of the monkey during thermoregulation. Brain Res *84*, 47-61.

Nash, J. F., Jr., Meltzer, H. Y., and Gudelsky, G. A. (1989). Selective cross-tolerance to 5-HT1A and 5-HT2 receptor-mediated temperature and corticosterone responses. Pharmacol Biochem Behav *33*, 781-785.

Naylor, L., Dean, B., Pereira, A., Mackinnon, A., Kouzmenko, A., and Copolov, D. (1998). No association between the serotonin transporter-linked promoter region polymorphism and either schizophrenia or density of the serotonin transporter in human hippocampus. Mol Med *4*, 671-674.

Nishio, H., Nezasa, K., and Nakata, Y. (1995). Role of calcium ion in platelet serotonin uptake regulation. Eur J Pharmacol *288*, 149-155.

Nunes, J. L., Sharif, N. A., Michel, A. D., and Whiting, R. L. (1991). Dopamine D2-receptors mediate hypothermia in mice: ICV and IP effects of agonists and antagonists. Neurochem Res *16*, 1167-1174.

O'Callaghan J, M. D. (1994). Neurotoxicity Profiles of Substituted Amphetamines in the C57Bl/6J Mouse. JPET *270*, 741-751.

Ogilvie, A. D., Battersby, S., Bubb, V. J., Fink, G., Harmar, A. J., Goodwim, G. M., and Smith, C. A. (1996). Polymorphism in serotonin transporter gene associated with susceptibility to major depression. Lancet *347*, 731-733.

O'Hearn, E., Battaglia, G., De Souza, E. B., Kuhar, M. J., and Molliver, M. E. (1988). Methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) cause selective ablation of serotonergic axon terminals in forebrain: immunocytochemical evidence for neurotoxicity. J Neurosci *8*, 2788-2803.

Oruc, L., Verheyen, G. R., Furac, I., Jakovljevic, M., Ivezic, S., Raeymaekers, P., and Van Broeckhoven, C. (1997). Association analysis of the 5-HT2C receptor and 5-HT transporter genes in bipolar disorder. Am J Med Genet *74*, 504-506.

O'Shea E, E. B., Camerero J, Green A, Colado M (2001). Effect of GBR 12909 and Fluoxetine on the acute and longterm changes induced by MDMA ('ecstasy') on the 5-Ht and dopamine concentrations in mouse brain. Neuropharmacology *40*, 65-74.

O'Shea, E., Granados, R., Esteban, B., Colado, M. I., and Green, A. R. (1998). The relationship between the degree of neurodegeneration of rat brain 5- HT nerve terminals and the dose and frequency of administration of MDMA ('ecstasy'). Neuropharmacology *37*, 919-926.

Owens, M. J., and Nemeroff, C. B. (1994). Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter. Clin Chem *40*, 288-295.

Page, I. H. (1976). The discovery of serotonin. Perspect Biol Med *20*, 1-8.

Penington, N. J., and Fox, A. P. (1994). Effects of LSD on Ca++ currents in central 5-HT-containing neurons: 5- HT1A receptors may play a role in hallucinogenesis. J Pharmacol Exp Ther *269*, 1160-1165.

Penington, N. J., and Kelly, J. S. (1990). Serotonin receptor activation reduces calcium current in an acutely dissociated adult central neuron. Neuron *4*, 751-758.

Penington, N. J., Kelly, J. S., and Fox, A. P. (1991). A study of the mechanism of Ca2+ current inhibition produced by serotonin in rat dorsal raphe neurons. J Neurosci *11*, 3594-3609.

Penington, N. J., Kelly, J. S., and Fox, A. P. (1993). Whole-cell recordings of inwardly rectifying K+ currents activated by 5- HT1A receptors on dorsal raphe neurones of the adult rat. J Physiol *469*, 387-405.

Pineyro, G., Blier, P., Dennis, T., and de Montigny, C. (1994). Desensitization of the neuronal 5-HT carrier following its long-term blockade. J Neurosci *14*, 3036-3047.

Pisani, F. M., Rella, R., Raia, C. A., Rozzo, C., Nucci, R., Gambacorta, A., De Rosa, M., and Rossi, M. (1990). Thermostable beta-galactosidase from the archaebacterium Sulfolobus solfataricus. Purification and properties. Eur J Biochem *187*, 321-328.

Price, L. H., Charney, D. S., Delgado, P. L., and Heninger, G. R. (1991). Serotonin function and depression: neuroendocrine and mood responses to intravenous L-tryptophan in depressed patients and healthy comparison subjects. Am J Psychiatry *148*, 1518-1525.

Qian, Y., Galli, A., Ramamoorthy, S., Risso, S., DeFelice, L. J., and Blakely, R. D. (1997). Protein kinase C activation regulates human serotonin transporters in HEK-293 cells via altered cell surface expression. J Neurosci *17*, 45-57.

Ramamoorthy, J. D., Ramamoorthy, S., Papapetropoulos, A., Catravas, J. D., Leibach, F. H., and Ganapathy, V. (1995a). Cyclic AMP-independent up-regulation of the human serotonin transporter by staurosporine in choriocarcinoma cells. J Biol Chem *270*, 17189-17195.

Ramamoorthy, S., Bauman, A. L., Moore, K. R., Han, H., Yang-Feng, T., Chang, A. S., Ganapathy, V., and Blakely, R. D. (1993a). Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization. Proc Natl Acad Sci U S A *90*, 2542-2546.

Ramamoorthy, S., and Blakely, R. D. (1999). Phosphorylation and sequestration of serotonin transporters differentially modulated by psychostimulants. Science *285*, 763-766.

Ramamoorthy, S., Giovanetti, E., Qian, Y., and Blakely, R. D. (1998). Phosphorylation and regulation of antidepressant-sensitive serotonin transporters. J Biol Chem *273*, 2458-2466.

Ramamoorthy, S., Leibach, F. H., Mahesh, V. B., and Ganapathy, V. (1993b). Partial purification and characterization of the human placental serotonin transporter. Placenta *14*, 449-461.

Ramamoorthy, S., Ramamoorthy, J. D., Prasad, P. D., Bhat, G. K., Mahesh, V. B., Leibach, F. H., and Ganapathy, V. (1995b). Regulation of the human serotonin transporter by interleukin-1 beta. Biochem Biophys Res Commun *216*, 560-567.

Rampin, O., and Giuliano, F. (2000). Central control of the cardiovascular and erection systems: possible mechanisms and interactions. Am J Cardiol *86*, 19F-22F.

Rioux, A., Fabre, V., Lesch, K. P., Moessner, R., Murphy, D. L., Lanfumey, L., Hamon, M., and Martres, M. P. (1999). Adaptive changes of serotonin 5-HT2A receptors in mice lacking the serotonin transporter. Neurosci Lett *262*, 113-116.

Rudnick, G., and Clark, J. (1993). From synapse to vesicle: the reuptake and storage of biogenic amine neurotransmitters. Biochim Biophys Acta *1144*, 249-263.

Rudnick, G., and Wall, S. C. (1992). The molecular mechanism of "ecstasy" [3,4-methylenedioxy- methamphetamine (MDMA)]: serotonin transporters are targets for MDMA- induced serotonin release. Proc Natl Acad Sci U S A *89*, 1817-1821.

Ruwe, W. D., and Myers, R. D. (1982). 5-HT receptors and hyper- or hypothermia: elucidation by catecholamine antagonists injected into the cat hypothalamus. Brain Res Bull *8*, 79-86.

Sabol, K. E., and Seiden, L. S. (1998). Reserpine attenuates D-amphetamine and MDMA-induced transmitter release in vivo: a consideration of dose, core temperature and dopamine synthesis. Brain Res *806*, 69-78.

Salmi, P. (1998). Independent roles of dopamine D1 and D2/3 receptors in rat thermoregulation. Brain Res *781*, 188-193.

Salmi, P., and Ahlenius, S. (1998). Evidence for functional interactions between 5-HT1A and 5-HT2A receptors in rat thermoregulatory mechanisms. Pharmacol Toxicol *82*, 122-127.

Salmi, P., Jimenez, P., and Ahlenius, S. (1993). Evidence for specific involvement of dopamine D1 and D2 receptors in the regulation of body temperature in the rat. Eur J Pharmacol *236*, 395-400.

Sanchez, C. (1989). The effects of dopamine D-1 and D-2 receptor agonists on body temperature in male mice. Eur J Pharmacol *171*, 201-206.

Sarhan, H., Grimaldi, B., Hen, R., and Fillion, G. (2000). 5-HT1B receptors modulate release of [3H]dopamine from rat striatal synaptosomes: further evidence using 5-HT moduline, polyclonal 5-HT1B receptor antibodies and 5-HT1B receptor knock-out mice. Naunyn Schmiedebergs Arch Pharmacol *361*, 12-18.

Scearce-Levie, K., Viswanathan ,S,Hen, R (1999). Locomotor response to MDMA is attenuated in Knockout mice lacking the 5-HT1B receptor. Psychopharmacology *141*, 154-161.

Schedl, A., Larin, Z., Montoliu, L., Thies, E., Kelsey, G., Lehrach, H., and Schutz, G. (1993a). A method for the generation of YAC transgenic mice by pronuclear microinjection. Nucleic Acids Res *21*, 4783-4787.

Schedl, A., Montoliu, L., Kelsey, G., and Schutz, G. (1993b). A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice. Nature *362*, 258-261.

Schildkraut, J. J. (1965). The catecholamine hypothesis of affective disorders: a review of supporting evidence. Am J Psychiatry *122*, 509-522.

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. Brain Res Bull *53*, 689-710.

Schnaitman, C. A., and Pedersen, P. L. (1968). Localization of oligomycin-sensitive ADP-ATP exchange activity in rat liver mitochondria. Biochem Biophys Res Commun *30*, 428-433.

Schroeter, S., and Blakely, R. D. (1996). Drug targets in the embryo. Studies on the cocaine- and antidepressant- sensitive serotonin transporter. Ann N Y Acad Sci *801*, 239-255.

Shaw, D. M., Camps, F. E., and Eccleston, E. G. (1967). 5-Hydroxytryptamine in the hind-brain of depressive suicides. Br J Psychiatry *113*, 1407-1411.

Sheard, M. H., and Aghajanian, G. K. (1967). Neural release of brain serotonin and body temperature. Nature *216*, 495-496.

Shen, S., Battersby, S., Weaver, M., Clark, E., Stephens, K., and Harmar, A. J. (2000a). Refined mapping of the human serotonin transporter (SLC6A4) gene within 17q11 adjacent to the CPD and NF1 genes. Eur J Hum Genet *8*, 75-78.

Shen, S., Spratt, C., Sheward, W. J., Kallo, I., West, K., Morrison, C. F., Coen, C. W., Marston, H. M., and Harmar, A. J. (2000b). Overexpression of the human VPAC2 receptor in the suprachiasmatic nucleus alters the circadian phenotype of mice. Proc Natl Acad Sci U S A *97*, 11575-11580.

Slikker, W., Jr., Holson, R. R., Ali, S. F., Kolta, M. G., Paule, M. G., Scallet, A. C., McMillan, D. E., Bailey, J. R., Hong, J. S., and Scalzo, F. M. (1989). Behavioral and neurochemical effects of orally administered MDMA in the rodent and nonhuman primate. Neurotoxicology *10*, 529-542.

Sonders, M. S., and Amara, S. G. (1996). Channels in transporters. Curr Opin Neurobiol *6*, 294-302.

Sora, I., Wichems, C., Takahashi, N., Li, X. F., Zeng, Z., Revay, R., Lesch, K. P., Murphy, D. L., and Uhl, G. R. (1998). Cocaine reward models: conditioned place preference can be established in dopamine- and in serotonin-transporter knockout mice. Proc Natl Acad Sci U S A *95*, 7699-7704.

Staley, J. K., Malison, R. T., and Innis, R. B. (1998). Imaging of the serotonergic system: interactions of neuroanatomical and functional abnormalities of depression. Biol Psychiatry *44*, 534-549.

Starke, K., Gothert, M., and Kilbinger, H. (1989). Modulation of neurotransmitter release by presynaptic autoreceptors. Physiol Rev *69*, 864-989.

Steinbusch, H. W. (1981). Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. Neuroscience *6*, 557-618.

Steinbusch, H. W., Verhofstad, A. A., Penke, B., Varga, J., and Joosten, H. W. (1981). Immunohistochemical characterization of monoamine-containing neurons in the central nervous system by antibodies to serotonin and noradrenalin. A study in the rat and the lamprey (Lampetra fluviatilis). Acta Histochem Suppl *24*, 107-122.

Stenfors, C., Yu, H., and Ross, S. B. (2000). Enhanced 5-HT metabolism and synthesis rate by the new selective r5- HT1B receptor antagonist, NAS-181 in the rat brain. Neuropharmacology *39*, 553-560.

Stenfors, C., Yu, H., and Ross, S. B. (2001). Pharmacological characterisation of the decrease in 5-HT synthesis in the mouse brain evoked by the selective serotonin re-uptake inhibitor citalopram. Naunyn Schmiedebergs Arch Pharmacol *363*, 222-232.

Stone, D., Hanson, G, Gibb ,J (1987). Differences in the central serotonergic effects of methylenedioxymetamphetamine(MDMA) in Mice and rats. Neuropharmacology *26*, 1657-1661.

Sugimoto, Y., Yamada, J., and Horisaka, K. (1991). Activation of peripheral serotonin2 receptors induces hypothermia in mice. Life Sci *48*, 419-423.

Svensson, T. H. (1978). Attenuated feed-back inhibition of brain serotonin synthesis following chronic administration of imipramine. Naunyn Schmiedebergs Arch Pharmacol *302*, 115-118.

Tamir, H., and Gershon, M. D. (1990). Serotonin-storing secretory vesicles. Ann N Y Acad Sci *600*, 53-66.

Tatsumi, M., Groshan, K., Blakely, R. D., and Richelson, E. (1997). Pharmacological profile of antidepressants and related compounds at human monoamine transporters. Eur J Pharmacol *340*, 249-258.

Tork, I. (1990). Anatomy of the serotonergic system. Ann N Y Acad Sci *600*, 9-34.

Twarog, B., M (1988). Serotonin : History of a discovery. Comp BiochemPhysiol *91*, 21-24.

Tyce, G. M. (1990). Origin and metabolism of serotonin. J Cardiovasc Pharmacol *16*, S1-7.

Ungerstedt, U. (1971). Stereotaxic mapping of the monoamine pathways in the rat brain. Acta Physiol Scand Suppl *367*, 1-48.

Uphouse, L. (2000). Female gonadal hormones, serotonin, and sexual receptivity. Brain Res Brain Res Rev *33*, 242-257.

Van Nueten, J. M., Leysen, J. E., de Clerck, F., and Vanhoutte, P. M. (1984). Serotonergic receptor subtypes and vascular reactivity. J Cardiovasc Pharmacol *6 Suppl 4*, S564-574.

Van Tienhoven, A., Scott, N. R., and Hillman, P. E. (1979). The hypothalamus and thermoregulation: a review. Poult Sci *58*, 1633-1639.

Vassaux, G., and Huxley, C. (1997). A dicistronic construct allows easy detection of human CFTR expression from YAC DNA in human cells. Nucleic Acids Res *25*, 4167-4168.

Vasse, M., Chagraoui, A., Henry, J. P., and Protais, P. (1990). The rise of body temperature induced by the stimulation of dopamine D1 receptors is increased in acutely reserpinized mice. Eur J Pharmacol *181*, 23-33.

Vialli, M., Erspamer, V. (1933). Cellule enterochromaffini e cellule basigranulose acidofile nei vertebrati. Z Zellforsch Mikrosk Ant *19*, 743.

Vitalis, T., Cases, O., Callebert, J., Launay, J. M., Price, D. J., Seif, I., and Gaspar, P. (1998). Effects of monoamine oxidase A inhibition on barrel formation in the mouse somatosensory cortex: determination of a sensitive developmental period. J Comp Neurol *393*, 169-184.

Wallace, J. A., and Lauder, J. M. (1983). Development of the serotonergic system in the rat embryo: an immunocytochemical study. Brain Res Bull *10*, 459-479.

Weissbach, H., Lovenberg,W.Redfield,B.G., Udenfield,S. (1961). *In vivo* metabolism of serotonin and tryptamine : effect of monoamine oxidase inhibition. J Pharmacol Exp Ther *131*, 26-30.

Westlund, K. N., Denney, R. M., Kochersperger, L. M., Rose, R. M., and Abell, C. W. (1985). Distinct monoamine oxidase A and B populations in primate brain. Science *230*, 181-183.

Whitaker-Azmitia, P. M. (1991). Role of serotonin and other neurotransmitter receptors in brain development: basis for developmental pharmacology. Pharmacol Rev *43*, 553-561.

Whitaker-Azmitia, P. M., Druse, M., Walker, P., and Lauder, J. M. (1996). Serotonin as a developmental signal. Behav Brain Res *73*, 19-29.

Wielosz, M., Salmona, M., de Gaetano, G., and Garattini, S. (1976). Uptake of 14C-5-Hydroxytryptamine by human and rat platelets and its pharmacological inhibition. A comparative kinetic analysis. Naunyn Schmiedebergs Arch Pharmacol *296*, 59-65.

Williams, J. T., Colmers, W. F., and Pan, Z. Z. (1988). Voltage- and ligand-activated inwardly rectifying currents in dorsal raphe neurons in vitro. J Neurosci *8*, 3499-3506.

Yamada, J., Sugimoto, Y., Wakita, H., and Horisaka, K. (1988). The involvement of serotonergic and dopaminergic systems in hypothermia induced in mice by intracerebroventricular injection of serotonin. Jpn J Pharmacol *48*, 145-148.

Yamada, J., Wakita, H., Sugimoto, Y., and Horisaka, K. (1987). Hypothermia induced in mice by intracerebroventricular injection of tryptamine: involvement of the 5-HT1 receptor. Eur J Pharmacol *139*, 117-119.

Zaczek, R., and Coyle, J. T. (1982). Rapid and simple method for measuring biogenic amines and metabolites in brain homogenates by HPLC-electrochemical detection. J Neural Transm *53*, 1-5.

Zahniser, N. R., Gerhardt, G. A., Hoffman, A. F., and Lupica, C. R. (1998). Voltage-dependency of the dopamine transporter in rat brain. Adv Pharmacol *42*, 195-198.

Zarrindast, M. R., and Tabatabai, S. A. (1992). Involvement of dopamine receptor subtypes in mouse thermoregulation. Psychopharmacology *107*, 341-346.

Zhou, F. C., Sari, Y., and Zhang, J. K. (2000). Expression of serotonin transporter protein in developing rat brain. Brain Res Dev Brain Res *119*, 33-45.

Zhou, F. C., Tao-Cheng, J. H., Segu, L., Patel, T., and Wang, Y. (1998). Serotonin transporters are located on the axons beyond the synaptic junctions: anatomical and functional evidence. Brain Res *805*, 241-254.